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Ottawa Hull K1A 0C9

(21) (A1) 2,158,655
(86) 1994/03/18
(43) 1994/09/29

(51) Int.Cl. [6] C12N 15/87; C12N 5/10; A61K 48/00; A61K 39/00; A61K 47/48

(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Process for Preparing Cancer Vaccines

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(30) (AT) 556/93 1993/03/19
(DE) P 43 26 821.8 1993/08/10

(57) 55 Claims

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(57) Abstract

Cancer vaccines are prepared by treating tumor cells or fibroblasts with a complex of immunostimulating polypeptide-coding DNA and of DNA-binding substance, for example polylysine, preferably conjugated with transferrine. The complex further contains a conjugate of DNA-binding substance and of an endosomolytic peptide or an adenoviras having at least one E4 defect or one E1a defect associated with other genetic defects.

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Process for preparing cancer vaccines

The invention relates to gene therapy, particularly its use in cancer therapy.

In the last 20 years there has been no crucial breakthrough in the treatment of cancers; only the recently developed formulation of the so-called "cancer vaccines" has given rise to hope of progress in cancer therapy.

In cancer patients the immune system has a considerable influence on the development of the tumour and the prognosis of the disease. In patients with malignant melanoma the immune response can lead to total regression of the tumour in 0.5% of patients. In most patients, on the other hand, an immune response which controls the development of the tumour but cannot switch off all the cancer cells is observed at early stages of the tumour. In advanced stages the situation is often found in which the cancer cells have escaped from immune recognition or the immune system is specifically depressed (Hoon et al., 1990). It has been proposed to administer cancer vaccines to the patient after surgical removal of the primary tumour. Cancer vaccines contain cancer cells which are genetically modified or cancer cells in conjunction with immunostimulating adjuvants which induce or reactivate a cancer-specific immune response in the patient (cf. for example Hoon et al., 1990; Bystryn et al., 1990 and 1992; Berd et al., 1990; Fearon et al., 1990; Lotze et al., 1992; Pardoll, 1992; Rosenberg et al., 1992, Schirrmacher, 1990).

The genes with which tumour cells are transfected in order to make them more immunogenic and consequently

less tumorigenic fall into three categories:
1) Genes which code for proteins which are lacking from the tumour cells (so-called foreign-or neoantigens): the so-called "xenogenisation" thus produced may, for example, by the expression of syngenetic MHC-I-antigens in MHC-I-deficient tumour cells, by expression of allogenetic MHC-I- or MHC-II-antigens or by expression of viral proteins such as haemagglutinin (Itaya et al., 1987; Fearon et al., 1988; Plaksin et al., 1988 and Ostrand-Rosenberg et al., 1990).
2) Cytokine genes: the expression of these genes (e.g. interleukin-2, CSF = "Colony stimulating factor", interferons) serves to activate the immune system so that it recognises the tumour cells as being foreign and rejects them.
3) Genes which code for so-called "auxiliary proteins" or "co-stimulatory molecules": very recent findings in immunology have shown that efficient stimulation of T-cells requires both activation of the T-cell receptor and also activation of a second receptor on the T-cell by a molecule on the surface of the antigen-presenting cell (Jenkins and Johnson, 1993). The pair B7/CD28 constitutes a unit of co-stimulating molecules of this kind. The expression of B7 on melanoma cells stimulates an immune response of normally non-immunogenic cells (Baskar et al., 1993; Chen et al., 1992; Townsend and Allison, 1993, Schwartz, 1992). The heat stable antigen HSA which is expressed, *inter alia*, by dendritic cells and spleen-B cells also exhibits a co-stimulating activity (Liu et al., 1992a and 1992b) and would appear to cooperate with B7 in promoting T-cell growth. One of the properties of co-stimulatory molecules such as B7 would appear to be that they impart properties of antigen-presenting cells to the tumour cells.

The cancer-specific immune response achieved by transfection of the tumour cells with a gene from one of

these groups is supposed to ensure that, after removal of the tumour, micrometastices which are clinically undetectable at present and cannot be removed surgically are destroyed in order to prevent cancer from reoccurring at a later date.

Therapy based on cancer vaccines, unlike non-specific stimulation of the immune system, constitutes an active-specific immune therapy with vaccines from inactivated tumour cells or parts thereof, if possible from the same patient, as a result of which the immune system of the patient is mobilised in a controlled manner against antigens of the individual tumour or at least the same type of tumour.

After it was found that inactivation of the tumour cells could result in a reduction in the immune response, recently cancer vaccines have been developed consisting of viable tumour cells (Rosenberg et al., 1992). However, for reasons of safety, it is desirable to have a vaccine based on cells which are no longer capable of dividing and which have a limited lifespan.

One of the critical steps in the production of cancer vaccines is the gene transfer into the cells which constitute the vaccine or a component thereof.

The most advanced technique hitherto developed for transferring genes into the cells, in the course of the use of cancer vaccines, *inter alia*, makes use of recombinant retroviral vectors; a method of this kind was recently proposed by Rosenberg et al., 1992. However, the use of retroviruses is problematic because it brings with it the risk of side effects such as infection with the virus, at least in a small percentage of cases. Moreover, retroviruses can only transduce dividing cells. Furthermore, these vectors, like the

recombinant adenoviruses proposed as an alternative to the retroviral system, are subject to restrictions regarding the size and construction of the DNA which is to be transferred.

Recently, a number of studies have proposed the use of non-recombinant adenoviruses on the basis of the ability of these viruses to liberate the contents of endosomes for gene transfer with DNA complexes using receptor-mediated endocytosis. The use of adenoviruses brings about an increase in the efficiency of gene transfer by avoiding the breakdown of the DNA complexes internalised in the cell into the lysosomes (Curiel et al., 1991; Curiel et al., 1992a; Zatloukal et al., 1992; Cotten et al., 1992; Wagner et al., 1992; Curiel et al., 1992b). It was proposed, *inter alia*, to modify the adenoviruses by binding to polylysine. The adenovirus-polylysine conjugates may be complexed with DNA, together with conjugates of transferrin-polylysine, to form ternary transferrin-polylysine/adenovirus-polylysine/DNA complexes (Wagner et al., 1992). The complexes bind to transferrin and adenovirus receptors on the target cells. After the endocytosis the adenovirus causes the endosomes to break open, resulting in the liberation of the material from the endosome into the cytoplasm. The DNA can then enter the cell nucleus, where the gene is expressed, predominantly by episomally localised DNA. This technique has the following advantages over conventional viral and non-viral gene transfer methods: since in this context the adenovirus acts only as an agent for liberating the transfection complexes from the endosome, the virus can be inactivated using genetic and/or chemical methods optionally in conjunction with physical methods, improving the level of safety compared with conventional viral techniques (Cotten et al., 1992). In addition, the gene construct is transported on the outside of the virus; it is not part of the virus

genome. Therefore, no viral vectors need to be prepared, and there are virtually no limitations as to the size and sequence of the DNA transported.

Another advantage of receptor-mediated gene transfer consists in the range of applicability with regard to the target cells. Thus, complexes which contain transferrin and adenovirus conjugates can be absorbed via transferrin and/or adenovirus receptors. Instead of transferrin, other ligands which are specific to certain cell populations may be used, e.g. LDL has proved very suitable for melanoma cells. Using this system high values can be achieved for gene expression in many types of cells (10 - 100 times higher than for retrovirally transfected cells (Lotze et al., 1992; Rosenberg et al., 1992) or for stably transfected cells obtained by $CaPO_4$ co-precipitation (Fearon et al., 1990)). In addition, multiple copies of the gene construct can be transported into the cells. Dividing and non-dividing cells can be transfected. The expression of the DNA transported into the cell is maintained for several months in confluent (growth-arrested) cell cultures (Zatloukal et al., 1992).

Apart from adeno- and other viruses or virus fragments, certain peptides also have the property of being able to break open the endosomes. Such peptides, which are also known as "endosomolytic" or "fusogenic" peptides have also been used to bring about an increase in gene expression during gene transfer using receptor mediated endocytosis. The use of such peptides for gene transfer is described in WO 93/07283.

The experiments carried out with the adenovirus dl312 have shown up problems of toxicity; the replication defect of the virus, which can be traced back to a defect in the E1A-region, could be partially

circumvented by the transfected cells, i.e. the defect is "leaky". In addition, the yield of virus in the packing cell lines which are available for complementing the defects of Ad5 dl312 was unsatisfactory.

The objective of the present invention was to provide a process for preparing cancer vaccines based on an improved gene transfer system.

The invention thus relates to a process for preparing cancer vaccines which contain autologous tumour cells. The process is characterised in that tumour cells or fibroblasts are cultivated and the cultivated cells are transfected *ex vivo* with a composition containing the following components:

ai) a DNA molecule which contains one or more sequences which can be expressed in the cell, which code for one or more, identical or different, immunostimulant polypeptides, or a plurality of DNA molecules, containing sequences coding for different immunostimulant polypeptides,

aii) optionally another DNA molecule which is free from sequences which code for a functionally active polypeptide in the cell which is to be transfected;

b) a conjugate between a DNA-binding molecule and an endosomolytically acting agent, selected from the group comprising
i) adenovirus, which has a mutation at least in the E4-region,
ii) adenovirus which has one of more other genetic defects in addition to an effect in the E1A-region, or
iii) endosomolytically active peptide;

optionally

c) a DNA-binding molecule, preferably conjugated with an internalising factor, which binds to a surface molecule of the cells to be transfected and is internalised in these cells,

components b) and c) together with the DNA defined in a) forming a substantially electroneutral complex, the transfected cells being inactivated in such a way that they lose their ability to divide whilst retaining their ability to express the DNA defined in ai), whilst in the case of the transfection of fibroblasts the latter are mixed with non-transfected and inactivated tumour cells, and the cell population is optionally mixed with pharmaceutically acceptable excipients and carriers.

The conjugate defined in b) is hereinafter referred to as "endosomolytic conjugate".

The cancer vaccines which may be obtained using the process according to the invention contain tumour cells inactivated with respect to the cancer vaccines proposed by Rosenberg et al., 1992, these tumour cells surprisingly being fully active in terms of the immune response which they trigger, in spite of their irradiation. This is presumably because, as a result of the high expression rates of the immunostimulant genes in the transfected cells, a reduction or loss of antigenicity caused by inactivation of the cells is at least partially compensated.

The starting material used for tumour vaccines prepared individually for each particular patient consists of autologous tumour cells, optionally mixed with fibroblasts. The fibroblasts may also be autologous cells, but it is also possible to use cells of a fibroblast cell line, thereby doing away with the need

to produce an individual fibroblast culture in each individual case, which would take several weeks.

First, tumour cells and/or fibroblasts are isolated from tissue samples from the individual to be treated. The methods used are known to those skilled in the art. Primary melanoma cells may most easily be isolated, for example, from lymph node metastases by surgically removing the metastases and dissociating them mechanically and optionally with additional enzymatic means, under sterile conditions.

Isolation from primary tumours is generally more difficult, particularly in the case of smaller tumours. For example, in the case of melanomas, the procedure is to remove the tumours surgically, grind them up mechanically and additionally dissociate them enzymatically. Known procedures for dissociation which can be used make use of collagenase, DNAse and hyaluronidase.

Isolation from other tumours may be carried out on the same principle; the methods of isolation and dissociation vary depending on the surrounding tissue. For the isolation and culture of tumour cells, it is possible to use methods known from the literature, such as those found in the textbook "Human Cancer in Primary Culture", Published by John R.W. Masters, 1991.

In a preferred embodiment of the invention the gene constructs are introduced, not into cancer cells but into primary fibroblasts which are then mixed with the non-transfected irradiated cancer cells which carry tumour antigens (Fakhrai et al., 1992). The advantage of this embodiment is that fibroblasts can be obtained easily and in large amounts from the patient, which is particularly important when smaller tumours are involved

and consequently a smaller number of tumour cells are available, and gene transfer into autologous primary fibroblasts is more easily standardised than transfection into primary cancer cell isolates from various patients. Another advantage of this embodiment is that the tumour cells do not have to be cultivated for a long period, with the result that their antigenic spectrum, which can be partially lost as a result of cultivation, is maintained. This also avoids the drawback connected with the cultivation of tumour cells, namely that individual populations, e.g. fibroblasts, which are contained in small amounts in the tumour isolate, or tumour cell clones, swamp the culture.

Fibroblasts are isolated from skin biopsies and cultivated using known methods. Such methods are described for example by Jones, 1989; Freshney, 1987; and Sly and Grubb, 1979.

After cultivation the cells are treated with the transfection medium which contains the complexes with components a) to c). Generally, conjugate b) and the complex between DNA and internalising factor conjugate are preferably administered simultaneously. In order to intensify gene expression, the complexes may also be administered repeatedly.

After transfection, the cells are freed from excess medium which contains transfection complexes, washed with fresh culture medium and further cultivated as required.

The inactivation of the tumour cells or fibroblasts which are preferably inactivated in the same way as the transfected fibroblasts, can be carried out using methods known *per se*, e.g. physical methods such as treatment with X-rays or gamma-rays, and/or by chemical

treatment with mitosis inhibitors, e.g. with mitomycin C. Suitable substances are those which block DNA replication or so-called "spindle toxins", namely substances which inhibit the mitosis spindle.

The suitable dosage for inactivation can be determined by, for example, determining the entry of ^{3}H-thymidine into the cells, on the one hand, or determining its rate of proliferation and, on the other hand, by measuring the expression of the foreign gene, at different dosages of radiation or concentrations of the chemical inactivating agent and/or different treatment times.

Irradiation of the transfected cells and the consequent restriction of their lifespan reduces any long-term side effects. A temporary, time-limited gene expression is achieved which is determined by the dosage of genes. In the course of the experiments carried out it was found that a dosage of gamma-rays of up to 100 Gy does not reduce the expression of the transfected gene constructs.

Preferably, the transfected fibroblasts are also inactivated. This procedure rules out any tumorigenicity which may have been acquired during transfection and/or culture.

The process for preparing the cancer vaccines preferably includes as an intermediate step, freezing of the cells under controlled conditions and with the addition of substances which avoid frost damage to the cells ("cryoprotectants"), such as dimethylsulphoxide (DMSO). The freezing step may in principle be provided at any stage of the process, e.g. before transfection of the cultivated cells or after transfection, but it is also possible to carry out the freezing as the last step, i.e. after the irradiation of the cells and immediately

before the vaccine is administered. The freezing provides a supply of cells which are ready for transfection or already transfected and this then makes it easier to prepare the tumour vaccines in aliquots. It is useful to have a frozen preparation which can be stored and need only be subjected to preparation, possibly including irradiation, before being used.

In any case, it is necessary to cleanse the cells from cryoprotectants before the tumour vaccines are administered.

The cancer vaccines prepared *in vitro* according to the process of the invention are administered to the patient in order to trigger or reactivate a systemic, tumour-specific immune response.

The quantity of tumour cells per immunisation is of the order of 10^5 - 10^7 cells. For the embodiment in which fibroblasts are cultivated and transfected, the number of fibroblasts added is in approximately the same range, but may be reduced to about 1/100 of the number of cells.

Within the scope of the present invention, trials were carried out on a mouse model to see how long after the injection of genetically altered melanoma cells, the cancer vaccines, these cells would be detectable at the injection site. Tests were also carried out to determine whether these cells are distributed in the blood or in different organs. Detection was carried out using polymerase chain reactions. During preparation of the cancer vaccines the cells were transfected with interleukin-2 plasmid DNA. Using specific primers, IL-2 and adenovirus DNA fragments were detected as markers for the genetically altered melanoma cells from the various tissue samples.

The tests at the immunisation site showed that the cancer vaccines administered, consisting of genetically changed melanoma cells, were eliminated within a few days of the injection. In the acute phase of cell degradation, 2 days after immunisation, it was shown that there was no transfer of the recombinant IL-2 DNA or adenovirus DNA to the blood, body organs or into the germ cells.

According to another aspect the invention relates to transfection complexes consisting of DNA containing one or more sequences coding for an immunostimulant polypeptide, a DNA-binding molecule which is preferably conjugated with an internalising factor for tumour cells and/or fibroblasts, particularly polylysine, and an endosomolytic conjugate consisting of a DNA-binding molecule and the above-defined adenovirus or a peptide, the DNA-binding molecule c) and the DNA-binding part of the conjugate b) being bound to the DNA.

It was found that, using the complexes according to the invention, it is possible to carry out efficient gene transfer into primary human melanoma cells and primary human fibroblasts, and that transfected mouse melanoma cells produced up to 24,000 units of IL-2 per 1 x 10^6 cells per 24 h, which is at least 30 times greater than the values achieved with other viral (Lotze et al., 1992; Rosenberg et al., 1992) or non-viral (Fearon et al., 1990) gene transfer techniques, and that irradiation of up to 100 Gy does not reduce the expression of the transfected gene constructs. Within the scope of the present invention the effect of a mouse melanoma vaccine was shown on a mouse model, this vaccine inducing a systemic immune response in immunised mice and protecting the animals from development of tumours after the administration of tumorigenic doses of melanoma cells. On the animal model, the effectiveness

of a melanoma vaccine against metastasis formation was also demonstrated.

The DNA molecule, defined as component ai), is a plasmid which contains a sequence in expressible form coding for an immunostimulant polypeptide, such as a cytokine. Within the scope of the present invention the term "immunostimulant" also includes the immune response-intensifying property of the so-called co-stimulatory molecules such as B7 (Schwartz, 1992; Townsend and Allison, 1993) or adhesion molecules such as HSAs ("heat stable antigens", Kay et al., 1990) or ICAM (Springer et al., 1987); immunostimulant substances also include foreign antigens (so-called "neo-antigens"), such as viral antigens. The sequence coding for the immunostimulant polypeptide is connected with regulatory sequences which permit the highest possible expression of the immunomodulatory polypeptide in the target cells. Preferably, strong promoters such as CMV-promoter (Boshart et al., 1985) or the β-actin-promoter (Gunning et al., 1987) are used. The appropriate construct can be determined in preliminary trials by comparing the expression values.

In a preferred embodiment of the invention the sequence coding for interleukin 2 (IL-2) is used.

However, it is also possible to use DNA sequences coding for other cytokines such as IL-4, IL-12, IFN-γ, TNF-α, GM-CSF (Pardoll, 1992). It is also possible to use combinations of cytokine sequences to intensify the immunostimulant activity, e.g. IL-2 + IFN-γ, IL-2 + IL-4, IL-2 + TNF-α or TNF-α + IFN-γ. Preferably, the sequences coding for two different cytokines are located on separate plasmids. In this way, as demonstrated, for example, in the experiments according to the invention using IL-2 and IFN-γ, fine gradation of the cytokine

expression can be obtained by varying the proportions of the two plasmids.

In another embodiment of the invention, a DNA molecule is used which contains one or more sequences coding for a co-stimulatory molecule. In a preferred embodiment the co-stimulatory molecule is the heat stable antigen (HSA). A DNA containing the sequence coding for HSA may be used both on its own and in conjunction with a DNA coding for a cytokine, preferably IL-2.

In another embodiment of the invention, a DNA molecule is used which contains one or more sequences coding for a neo-antigen. In a preferred embodiment the neo-antigen is a virus protein or a fragment thereof, such as the rabies glycoprotein. A DNA containing the sequence coding for the virus protein may be used both on its own and in conjunction with a DNA coding for a cytokine, preferably IL-2.

With regard to the size of the DNA construct there is virtually no limit; the gene transfer system used has proved suitable for constructs of a size of 48 kb (Cotten et al., 1992).

The DNA coding for an immunostimulant polypeptide, i.e. the therapeutically active DNA, may possibly occur in admixture with a DNA molecule as defined in aii) which serves as the "filler-DNA". The sequence of this DNA is not critical; apart from requirements as to purity, in which it should correspond to the therapeutically active DNA, it need only satisfy the condition of not containing any sequence which codes for a polypeptide which is functionally active in the cell. The size of this DNA is not critical either; generally, it is advisable for it to be of the same order of magnitude as the gene-therapeutically active DNA molecule or less

than the latter.

This filler-DNA may replace different amounts of the therapeutically active DNA, starting from a constant amount of DNA with respect to a defined ratio between the other partners of the complex. The advantage of this is that the therapeutic DNA dose and hence the amount of cytokine expressed in the cell can be varied without having to alter the other parameters, which is extremely beneficial within the scope of standardised tumour vaccine production. Within the scope of the present invention it has been demonstrated that the amount of gene expressed in the cell decreases in proportion to the amount of filler-DNA.

In another preferred embodiment of the present invention the plasmid DNA used is free from lipopolysaccharides. It has, surprisingly, been found that the expressed values are significantly increased if the plasmid DNA is substantially free from lipopolysaccharides. These constitute a frequent contaminant of plasmid DNA which is generally propagated in *E. coli*. In order to remove lipopolysaccharides, either the DNA may be purified by suitable methods, e.g. by a combination of chromatographic methods including polymyxin chromatography, or, in order to pick up also the lipopolysaccharides which may originate from the medium, lipopolysaccharide-binding reagents such as polymyxin may be added to the transfection medium.

If the target cell has receptors for the adenovirus which ensure internalisation in an amount which is sufficient for the efficient expression of the foreign DNA in the cell, it may be enough, when using adenovirus conjugates as component b), to use a DNA-binding molecule which is not conjugated with another internalising factor as component c); generally it will

be the same molecule as the one contained in conjugate b); polylysine is preferably used. For this embodiment of the invention the adenovirus itself acts as the internalising factor and in this case it is not necessary to conjugate the DNA-binding substance with another internalising factor.

In the embodiment of the invention in which c) is a non-conjugated DNA binding substance the DNA is complexed with conjugate b); in this case component c) serves primarily to bring about compacting and hence improved uptake of the complexes in the cell (Wagner et al., 1991a). For specific applications, e.g. when using small DNA molecules, a non-conjugated DNA-binding molecule as component c) may be omitted altogether.

In a preferred embodiment of the invention the DNA binding substance defined as component c) is conjugated with an internalising factor. This embodiment of the present invention is used particularly when, where an adenovirus conjugate is used, the target cell has no or only a few receptors for the adenovirus, e.g. when a virus from a remote species is used, or when a peptide conjugate is used as the endosomolytic conjugate b). In the presence of another internalising factor conjugate, the virus conjugates profit from the internalising capacity of conjugate c), by being complexed to the nucleic acid together with the latter conjugate c), and being absorbed into the cell as a component of the resulting complex, hereinafter referred to as the "combined complex" or "ternary complex". Without wishing to be tied to this theory, the combined complexes are absorbed by cells either by binding to the surface receptor which is specific to the internalising factor or by binding to the virus receptor or by binding to both receptors via receptor-mediated endocytosis. When the viruses are released from the endosomes the DNA

contained in the complexes is also released into the cytoplasm, thus escaping the lysosomal breakdown.

The conjugates preferably contained as c) between an internalising factor and a DNA-binding substance are known *per se*.

The phrase "internalising factor" for the purposes of the present invention includes ligands or fragments thereof which, after binding to a tumour cell or a fibroblast are internalised by endocytosis, preferably receptor-mediated endocytosis, or factors the binding/internalising of which is carried out by fusion with cell membrane elements.

Examples of internalising factors are the ligands transferrin (Klausner et al., 1983), asialoglycoproteins (such as asialotransferrin, asialorosomucoid or asialofetuin), (Ashwell et al., 1982), lectins (Goldstein et al., 1980; Sharon, 1987) or substances which contain galactose and are internalised via the asialoglycoprotein receptor; mannosylated glycoproteins (Stahl et al., 1978), lysosomal enzymes (Sly et al., 1982), LDL (Goldstein et al., 1982), modified LDL (Goldstein et al., 1979), lipoproteins which are absorbed into the cell via receptors (apo B100/LDL); viral proteins; antibodies (Mellman et al., 1984; Kuhn et al., 1982; Abrahamson et al., 1981) or fragments thereof against cell surface antigens, e.g. anti-CD4, anti-CD7, anti-CD3; cytokines such as interleukin-1 (Mizel et al., 1987), interleukin-2 (Smith et al., 1985), TNF (Imamura et al., 1987), interferons (Anderson et al., 1982); CSF ("Colony-stimulating Factor"), (Walker et al., 1987), factors and growth factors such as insulin (Marshall, 1985), EGF ("Epidermal Growth Factor"), (Carpenter, 1984); PDGF ("Platelet-Derived Growth Factor"), (Heldin et al., 1982), TGFα (Massague

et al., 1986) and TGFβ ("Transforming Growth Factors" α, β), HGF (Hepatocyte Growth Factor (Nakamura et al., 1989), nerve growth factor (Hosang et al., 1987), insulin-like growth factor I ("Insulin-like Growth Factor"), (Schalch et al., 1986), LH, FSH (Ascoli et al., 1978), growth hormone (Hizuka et al., 1981), prolactin (Posner et al., 1982), glucagon (Asada-Kubota et al., 1983), thyroid hormone (Cheng et al., 1980), α-2-macroglobulin-protease (Kaplan et al., 1979). Other examples are immunoglobulins or fragments thereof as ligands for the Fc-receptor or anti-immunoglobulin antibodies which bind to sIgs ("Surface Immunoglobulins"). The ligands may be of natural or synthetic origin (cf. Trends Pharmacol. Sci., 1989, and the references cited therein).

Transferrin and EGF are the preferred internalising factors within the scope of the present invention.

Suitable DNA-binding substances as component c) (non-conjugated or as conjugate component of an internalising factor conjugate) or as part of conjugate b) include, for example, homologous organic polycations such as polylysine, polyarginine, polyornithine or heterologous polycations with two or more different positively charged amino acids, whilst these polycations may have different chain lengths, as well as non-peptidic synthetic polycations such as polyethyleneimine. Other suitable DNA-binding substances are natural DNA-binding proteins of a polycationic nature such as histones or protamines or analogs or fragments thereof, as well as spermine or spermidine.

The length of the polycation is not critical provided that the complexes are substantially electroneutral. If the DNA consists of 6,000 bp and 12,000 negative charges the quantity of polycation per mol of DNA may be, for

example:

60 Mol Polylysine 200 or
30 Mol Polylysine 400 or
120 Mol Polylysine 100, etc.

The average man skilled in the art can select other combinations of polycation length and molar amounts by simple routine experiments.

Preferably, polylysine is used for the purposes of the present invention, more particularly with a chain length of about 200 to 300 lysines.

If, for the purpose of preparing the conjugates b), the DNA-binding molecule is modified with respect to coupling with the endosomolytic agent, particularly the virus, e.g. if polylysine is conjugated with streptavidin in order to be bound to biotinylated adenovirus, for simplicity's sake the DNA binding molecule thus modified may be used as component c). In practice, this means that an excess of polylysine-streptavidin is used, to prepare polylysine-streptavidin/biotin-adenovirus conjugates, this excess taking over the function of component c).

The internalising factor-polycation-conjugates may be prepared by chemical methods or, if the polycation is a polypeptide, by the recombinant methods; for methods of preparation we refer to the disclosure of EP 388 758.

The conjugates may also be prepared using the method described by Wagner et al., 1991b, in which a glycoprotein such as transferrin and the DNA-binding molecule, particularly polylysine, are bound together via one or more carbohydrate chains of the glycoprotein.

Preferably, an adenovirus conjugate b) contains an

adenovirus having a defect in the E4-region. Adenoviruses of this kind were described by Bridge and Ketner, 1989. The function of the proteins coded in the complex E4-region has only been partly explained. So far, it is known that the E4-region like the E1b region, is essential for the transition between the early and late gene expression programmes of the virus and for switching off the host protein synthesis and is also essential for virus replication and virion assembly. There are 24 E4-mRNAs, and hitherto seven open reading frames have been identified, the assumption being that reading frames 3 and 6 would be primarily responsible for the E4-phenotype. The gene product of the open reading frame ORF 6/7 has a very important function. This protein probably binds the cellular transcription factor E2F, whereupon the latter becomes the highly specific adenoviral transcription factor. It has, surprisingly, been found that an adenovirus which has a defect in the E4-region, has, as a component of a gene transfer system based on receptor mediated endocytosis, in which the adenovirus acts as an agent to break up the endosomes, when used on tumour cells, low toxicity with at the same time a high gene transport efficiency.

From the adenoviruses described by Bridge and Ketner, the mutant dl1014 which has an intact ORF 4 but wherein ORF 3 and ORF 6 as well as ORF 6/7 are defective was chosen for its quality of being most affected in the synthesis of viral proteins. Since the functions of the virus proteins coded in E4 have not yet been entirely clarified it is impossible to define at present the reading frames in which the mutations must be placed in order to achieve the desired effect. Other suitable mutants apart from mutant dl1014 can be determined empirically by preparing E4-mutants as described by Bridge and Ketner and testing them in standardised transfection and cytotoxicity experiments as described

in the Examples. For the guide transfection tests, first of all, a reporter gene may be used instead of the cytokine gene. Mutants which deliver comparable gene expression and cytotoxicity values to those of dl1014 or are even superior to dl1014 are suitable as components of the transfection complex within the scope of the present invention provided that they also satisfy the requirement that the viruses, such as dl1014 in cell line W162, can be cultured to high titres in a cell line which complements the E4-defect.

As an alternative to the adenovirus with an E4-defect, it is also possible to use an adenovirus with a defect in the E1a-region which in addition to the E1a-defect has one or more other genetic defects which are put in place using chemical, chemical/physical or genetic methods.

The defects established using chemical or physical/chemical methods are not targeted defects but may be scattered throughout the entire genome of the virus.

Within the scope of the present invention, an adenovirus mutant designated dl312 (Jones and Shenk, 1979) inactivated with 8-methoxypsoralen/UV has, *inter alia*, proved suitable for the preparation of cancer vaccines.

Other suitable E1a-mutants may be prepared using methods known from the literature, additionally treated with various inactivation methods and/or doses and tested, as described for E4-mutants, as to their suitability in preparing tumour vaccines as a component of the transfection complexes.

As an alternative to 8-methoxypsoralen, other psoralen derivatives may be used, e.g. 4'-aminomethyl-4,5',8-

trimethylpsoralen, which has been found to be suitable for the inactivation of adenoviruses in view of its use for gene transfer. (Psoralen derivatives have the ability to intercalate into the DNA and, after irradiation with UV at 365 nm, to form covalent inter-strand and intra-strand adducts with the virus DNA (Hanson, 1992). These adducts inactivate the affected gene sections and thereby block the gene functions, e.g. the transcription and replication of the virus.) It has been found that reduction of viral replication by more than 5 log (determined by CPE-assay) and a reduction of viral replication of more than 7 log (determined by plaque-assay) is accompanied by a reduction in the gene transfer amplification of only half of one power of ten (Cotten et al., 1992).

With respect to the maximum possible standardisation which is sought in the manufacture of drugs in general and in the manufacture of tumour vaccines in particular, this standardisation extending to all components and parameters of the process, i.e. to the viruses as well, it is advantageous to use a virus which has been inactivated, if possible, using a standardisable method. The need for standardisation is met particularly by chemical methods of inactivation which are consequently superior to the chemical/physical methods in this respect. One example of a chemical inactivation method is inactivation using β-propiolactone (Morgeaux et al., 1993; De Shu et al., 1986; Budowsky and Zalesskaya, 1991). This method has the advantage that there is no need to purify unreacted reagent owing to the instability of the inactivation agent in aqueous solutions. Moreover, this method of inactivation does not require any UV treatment, which is also advantageous in terms of standardisation. It has been established, within the framework of the present invention, that the treatment of adenovirus with two aliquots of 0.3%

β-propiolactone for 4 hours at ambient temperature causes a drop in the virus titre of 5 log, which is comparable with the inactivation achieved using 8-methoxypsoralen. The DNA transporting activity is maintained at average doses of β-propiolactone (0.3%), whereas treatment with 1% β-propiolactone causes a considerable loss of this ability. Analysis of the gene expression of the inactivated virus showed that both the psoralen derivatives and also β-propiolactone block virus gene expression (E1a and E3) to the same extent. The more sensitive plaque assay, however, showed that the psoralen treatment inactivates the virus by more than 7 log, with no plaques being observed at the highest doses of virus. By contrast, β-propiolactone inactivation only caused a drop in the virus titre of 5 log, with plaques visible at higher doses of virus.

Thus, in another embodiment of the invention, a virus is used which is inactivated using only chemical methods, preferably using β-propiolactone.

The suitability of a method for inactivating viruses with respect to their use for gene transfer, particularly for the tumour vaccines according to the invention, can be determined in preliminary trials, as illustrated in the examples for the inactivation of adenoviruses with 8-methoxypsoralen/UV, 4'-aminomethyl-4,5',8-trimethylpsoralen/UV and β-propiolactone. The efficiency of the virus inactivation is determined, for example, by measuring the virus titre and/or by means of the more sensitive plaque assay and in addition the ability of the virus to amplify the receptor mediated gene transfer is tested using a reporter gene. In order to find out which part of the virus genome is attacked by the inactivation method, i.e. which sections are destroyed, hybridisation tests can be carried out using DNA probes by means of which the viruses can be

investigated for the presence of the corresponding transcripts. Within the scope of the present invention, a number of inactivation methods were tested on this principle to see whether they are capable of blocking the virus replication and transcription functions. A method of inactivation is suitable if it delivers adenovirus particles which still have the endosomolytic activity (which is a function of the virus capsid) which is useful for the DNA transporting function, whilst they lack the ability to express or replicate the virus gene, which could give rise to undesirable changes in the cell. It has been found that the treatment of adenovirus with 8-methoxypsoralen or with 4'-aminomethyl-4,5',8-trimethylpsoralen, in each case followed by 25 minutes of UVA irradiation, as well as treatment with 2 x 0.3% β-propiolactone, yields virus particles which retain their ability to intensify efficient DNA transporting. Using CPE (cytopathic end point assay) it was found that the three treatment methods are comparable in terms of the decrease in the virus titre. Both after treatment with 8-methoxypsoralen/UV and also with β-propiolactone there was no detectable transcription of the virus genome. However, the more sensitive plaque assay showed that β-propiolactone-treated viruses are able to replicate if sufficient quantities of the complementing cell line are present. By contrast, treatment of the virus with both psoralen derivatives blocks the virus replication completely, so that using this test no plaques were detectable.

The RNA analysis for detecting gene expression was carried out either with an adenovirus 5 E1a probe, which recognises part of the E1a gene which is deleted in the adenovirus dl312, or with an adenovirus 5 E3 probe which recognises a part of the E3 region which codes for the abundant E3 19K glycoprotein (E1a acts as the control;

there should be no RNA signal in the virus dl312 but a signal should be detectable in dl1014, as it has a wild-type E1 region). From the expression of E3 it is assumed that it might play an important part in the immune response to transfected cells, since at least two of the E3 genes modulate the surface expression of MHC class I molecules and TNF receptor molecules on the surface of the infected cells. For use of the virus dl1014 in gene therapy, in which immunogenic tumour cells are produced, it is therefore desirable to have a defect in the E3 region, since the expression of this region in this context could interfere with the immune response to transfected cells.

The polylysine-coupled (or ionically bound) endosomolytic agent is preferably used as component of a ternary or combined complex. The coupling of adenovirus to polylysine may be carried out in various ways:

The coupling of the virus by a chemical method can be carried out in a manner known per se for the coupling of peptides, if necessary providing the individual components with linker substances before the coupling reaction (this procedure is necessary if there is no functional group suitable for coupling, e.g. a mercapto or alcohol group, already present). The linker substances are bifunctional compounds which are first reacted with functional groups of the individual components, after which the modified individual components are coupled.

One possible method of coupling the virus to polylysine is carried out in a similar way to the production of transferrin-polylysine conjugates (Wagner et al., 1990) after modification of the defective adenovirus using a heterobifunctional reagent. If a virus has suitable carbohydrate chains, it may be connected to the DNA-

binding substance via one or more carbohydrate chains of the glycoprotein (Wagner et al., 1991b).

Another method of preparing the virus-polylysine conjugates is by enzymatic coupling of the virus to a DNA-binding substance, particularly a polyamine, using a transglutaminase (Zatloukal et al., 1992).

Another method of preparing the adenovirus-polylysine conjugates consists in coupling the virus to the polycation via a biotin-protein bridge, preferably a biotin-streptavidin bridge (Wagner et al., 1992).

If desired, the binding to biotin may also be effected using avidin.

It is also possible to establish the bond between the virus and the polylysine by, on the one hand, biotinylating the virus and, on the other hand, conjugating an anti-biotin antibody with polylysine and establishing the bond between virus and polylysine via the biotin/antibody bond, using standard commercial polyclonal or monoclonal antibodies against biotin.

The bond between the virus and polylysine may also be produced by coupling polylysine with a lectin which has an affinity for a virus surface glycoprotein, the bonding being carried out in a conjugate of this kind by means of the bond between the lectin and the glycoprotein. If the virus has no suitable carbohydrate side chains of its own, it may be modified accordingly.

The virus may also be ionically bound to the DNA-binding molecule if it has, on its surface proteins, regions which are acidic and are therefore capable of binding to a polycation.

Apart from selected adenovirus mutants the endosomolytic component of the conjugates defined in b) may also be a peptide which is bound covalently or by ionic binding to a DNA-binding molecule. With regard to the demands made of such peptides and their coupling to the DNA-binding molecule, reference is made to WO 93/07283.

Preferably, within the scope of the present invention, a synthetic dimeric influenza peptide, ionically bound to polylysine, is used by means of which it is possible to achieve high expression values for IL-2 in melanoma cells. Melanoma cells transfected with IL-2-plasmid DNA in the presence of this peptide conjugate using transferrin-polylysine conjugates proved to be cancer vaccines with a prophylactic protective effect against tumour formation.

As for the preparation of the transfection complexes the sequence of the steps is not critical; the following procedure may be used, for example:

Starting from the DNA molecules, the correct type and quantity of DNA binding substance are determined for ensuring complexing of the DNA; the complexes obtained are preferably substantially electroneutral. For the complexes which contain the virus conjugate and an internalising factor conjugate, the cation part of both conjugates is taken into consideration with regard to the aspect of electroneutrality.

When determining the molar ratio of components a), b) and c) it should be borne in mind that complexing of the DNA takes place and care should be taken to ensure that the complex formed is bound to the cell, conveyed into the cell and liberated from the endosomes.

The ratio of internalising factor conjugate/DNA selected

depends primarily on the size of the polycation molecules and on the number and distribution of the positively charged groupings, criteria which are adapted to the size and structure of the DNA to be transported, whilst the virus conjugate should also be taken into account. Preferably, the molar ratio of internalising factor:polylysine is about 10:1 to about 1:10.

When using adenovirus conjugate and non-conjugated DNA binding substance, after determining the optimum ratio of conjugate:DNA for the efficiency of transfection and expression, using titration, it is possible to determine the quantity of the conjugate part which can be replaced by DNA-binding substance.

Preferably, polylysine is used both as component c), preferably conjugated with an internalising factor, and also as a part of the adenovirus conjugate b).

One suitable method of determining the ratio of the components contained in the complexes consists first in defining the gene construct which is to be introduced into the cell and, as described above, finding a virus which is suitable for transfection. Then the virus is bound to the polycation and complexed with the gene construct. Starting from a constant amount of DNA, the optimum ratio between virus conjugate and internalising factor conjugate or non-conjugated DNA-binding substance is determined by titration.

The complexes may be prepared by mixing together components a), b) and c), which are each present in the form of dilute solutions.

The optimum ratio of DNA to conjugate and non-conjugated DNA-binding substance is determined by titration, i.e. in a series of transfection experiments with a constant

quantity of DNA and a variable amount of conjugate/polylysine. The optimum ratio of conjugate to polycation in the mixture may be obtained using routine experiments or by comparing the optimum ratios of the mixtures used in the titration experiments.

The DNA complexes may be prepared at physiological saline concentrations. Another possibility is to use high salt concentrations (about 2 M NaCl) and then adjust to physiological conditions by slow dilution or dialysis.

The most suitable sequence for mixing the components is determined in preliminary trials for each individual case.

The quantity of endosomolytic conjugate used depends on the particular transfection being carried out. It is advisable to use the minimum amount of endosomolytic conjugate necessary to ensure internalisation of the complex into a majority of the cells and release thereof from the endosomes. If adenovirus conjugate is used, the quantity of conjugate is adapted to the particular type of cell, and in particular the infectiousness of the virus for this type of cell should be taken into consideration. Another criterion is the particular internalising factor conjugate, particularly with regard to the internalising factor for which the target cell has a specific number of receptors. In addition, the quantity of endosomolytic conjugate depends on the amount of DNA to be imported. For special applications, the optimum concentration of endosomolytic conjugate is determined by titration in preliminary trials using the target cells envisaged for transfection and the vector system envisaged for transfection, and appropriately using as DNA a gene construct which largely coincides with the one intended for actual use, in terms of its

size, and which contains a reporter gene for ease of measurement of the efficiency of gene transfer. Within the scope of the present invention, it has been shown that the luciferase gene is suitable as the reporter gene for these trials.

According to another aspect the invention relates to the tumour cells or fibroblasts transfected with the complexes according to the invention.

In another aspect the present invention relates to cancer vaccines which can be obtained by means of the process according to the invention. These vaccines are pharmaceutical preparations containing the transfected tumour cells or the transfected fibroblasts in admixture with tumour cells in a pharmaceutically acceptable formulation.

The cancer vaccines ready for use are preferably in the form of a suspension which is optionally obtained by trypsinisation. The cells are suspended in a physiological medium (physiological saline or buffer solution) which optionally contains those nutrients, particularly amino acids, which the cells require in order to maintain their metabolism for the short period between the preparation of the vaccine and its administration.

In the experiments carried out within the scope of the present invention, the melanoma cells were in RPMI 1640 medium with foetal calves serum (FCS) added thereto.

For galenic formulation of the tumour vaccines according to the invention, various media were tested with respect to their therapeutic use.

It was found that cell culture media containing

additives such as calf serum, human serum, human serum albumin and/or hydroxyethylcellulose ensure the viability of a number of cells sufficient for the vaccine. In terms of the maximum compatibility the tumour vaccines are preferably in serum of blood group AB, and it is also possible to use autologous serum as the medium. If desired, the medium contains added amounts of growth factors or cytokines such as IFN-gamma or GM-CSF, in order to have a favourable effect on the antigen presentation of the cells.

The type of cancer vaccines which can be prepared by means of the present invention constitutes a new development in the field of cytokine therapy. It has the advantage that the severe side effects of systemically administered cytokines can be avoided because of the locally restricted production and effect of the cytokine. Apart from the treatment of melanomas and colon carcinomas, other types of cancer may also be treated with the cancer vaccines according to the invention, e.g. kidney cancer or breast cancer.

Summary of Figures

Fig. 1: Gene transport into primary human melanoma cell cultures

Fig. 2: Determination of the ligands involved in the uptake of gene transfer complexes. Use of non-conjugated polylysine

Fig. 3: Effect of the inactivation of adenoviruses by psoralen/UV on gene expression in primary human melanoma cells

Fig. 4: Determining the long-term cytotoxicity of adenoviruses

Fig. 5: Effect of the irradiation of tumour cells on the expression of transfected genes

Fig. 6: Influence of the plasmid concentration on the expression of the luciferase reporter gene

Fig. 7: Influence of the plasmid concentration on the expression of IL-2 and/or IFN-γ

Fig. 8: Loss of the tumorigenicity of transfected mouse melanoma cells

Fig. 9: Induction of a systemic immune response against melanoma in mice by immunisation with cytokine-transfected, irradiated tumour cells

Fig. 10: EGF as a ligand for gene transfer into cells of a human epidermoid carcinoma cell line (A: plus 2% FCS, B: without FCS)

Fig. 11: IL-2 expression in mouse melanoma cells using various vectors

Fig. 12: IL-2 expression in fibroblasts using various vectors

Fig. 13: Stimulation of an immune response by melanoma cells which express HSA on their surface

Fig. 14: Stimulation of an immune response by melanoma cells which express the rabies glycoprotein on their surface

Fig. 15: Titration of 8-methoxypsoralen

Fig. 16: Titration of 4'-aminomethyl-4,5',8-trimethyl-psoralen

Fig. 17: Titration of β-propiolactone

Fig. 18: Gene transporting activity of adenovirus dl1014 after treatment with 8-methoxypsoralen or after treatment with low concentrations of β-propiolactone

Fig. 19: Plaque assays of adenovirus dl1014, inactivated by 8-methoxypsoralen, β-propiolactone or 4'-aminomethyl-4,5',8-trimethylpsoralen

Fig. 20: Plaque assays of adenovirus dl1014, inactivated with 8-methoxypsoralen or various β-propiolactone treatments

Fig. 21: Gene expression of the inactivated virus

Fig. 22: Reverse transcription-PCR analysis

Fig. 23: Locating the deposit of 8-methoxypsoralen in the virus genome

Fig. 24: Growth of human melanoma cells depending on the dose of gamma-rays

Fig. 25: Protective effect against metastasis formation by immunising with cytokine-transfected melanoma cells (transfection by means of adenovirus)

Fig. 26: Effectiveness of the cancer vaccines depending on the cytokine dose

Fig. 27: Protective effect against metastasis formation by immunising with cytokine-transfected melanoma cells (transfection by means of endosomolytic peptide)

Fig. 28: Interleukin-2 expression in human melanoma cells

Fig. 29: Influence of the endotoxin content of the DNA on the expression of IL-2 in human melanoma cells

In the following Examples, which illustrate the present invention, the following materials and methods were used unless otherwise specified:

a) Plasmid constructs

i) DNA plasmid constructs containing the sequence coding for human or murine IL-2

For the experiments carried out in Examples 6 to 8 the plasmid BCMGneo-mIL-2 described by Karasuyama et al., 1989 was used.

Alternatively a plasmid designated pWS2m was prepared which contains the murine IL-2 gene under the control of the cytomegalovirus enhancer/promoter: the plasmid

pHβAPr-1 (Gunning et al., 1987) was cut with BamHI and EcoRI. By agarose gel purification a 2.5 kb fragment was isolated which contains the ampicillin resistance gene and the replication origin of pBR322 as well as the SV40-polyadenylation signal. This fragment was ligated with the CMV-promoter/enhancer, which was amplified as a 0.7 kb PCR fragment of vector pAD-CMVI (described in EP-A 393 438) and digested with EcoRI/BamHI. The plasmid contained was named pWS. The cDNA coding for murine IL-2 was obtained as a PCR fragment from plasmid BMGneo-mIL-2 (Karasuyama and Melchers, 1988). The sequence GCCGCC was attached in the direction of the 5'-end from the first ATG codon for optimum initiation of translation. The 3'-non-coding region was removed. The PCR fragment was ligated into the vector pWS opened by SalI/BamHI digestion.

For use in human cells, the vector pWS2 is used, which is obtained in a similar way to the vector pWS2m, except that, instead of the plasmid BMGneo-mIL-2, the plasmid pIL2-50A (Taniguchi et al., 1983) which contains the cDNA coding for human IL-2 is used as the basis for PCR amplification.

As an alternative to a plasmid with the ampicillin resistance gene, the vector pGShIL-2tet was prepared which contains the tetracyclin resistance gene excised from pBR327.

For the plasmid pCM2 the murine IL-2 sequence was excised from BCMGneo-mIL-2 together with the 5'- and 3'-flanking regions (cleavage and poly(A) signals from the rabbit β-globin gene) as an SalI/BamHI fragment and inserted in the vector pAD-CMV1.

ii) DNA plasmid construct containing the sequence coding for murine IFN-γ

The plasmid pSVEmuIFN-γ described by Gray and Goeddel, 1983 was used.

iii) DNA plasmid construct pHSA, containing the sequence coding for HSA

The plasmid pHSA which contains the HSA sequence driven by the CMV-promoter was obtained by cloning the HSA-cDNA (Kay et al., 1990) into the BstXI site of the plasmid pCDM8 (Seed, 1987).

iv) DNA plasmid construct pWS-RABIES, containing the sequence coding for the rabies glycoprotein

The sequence coding for the rabies glycoprotein was isolated from the vector pKSV-10 (Pharmacia) which contains the rabies glycoprotein cDNA (Anilionis et al., 1982) cloned into the BglII site, downstream from the SV40-promoter, as a BglII fragment. The vector pWS2m was cut with BglII and BamHI. The fragment carrying the murine IL-2 cDNA was separated via agarose gel electrophoresis and the fragment corresponding to the vector pWS was isolated and ligated with the rabies fragment. The correct rabies orientation was confirmed by sequencing. The rabies glycoprotein sequence is shown as SEQ ID NO:1.

v) DNA plasmid construct pWS-Gm, containing the sequence coding for murine GM-CSF

The vector pWS2 described in i) was cut with SalI and BamHI and the fragment coding for IL-2 thus liberated was separated off by agarose gel electrophoresis. The DNA coding for murine GM-CSF was prepared totally synthetically from 12 mutually overlapping oligonucleotides. The sequence used corresponded to the sequence described by Miyatake et al., 1985 (coding area

from position 32 to position 457) except for silent exchanges in position 178 (A instead of G), position 274 (C instead of G) and position 355 (C instead of G). In addition, the triplet AGC (position 446 to 448) was replaced by GTC. The 5'-area was provided with an SalI-compatible overhang and the 3'-region with a BamHI-compatible overhang. The 5'-end was provided with a GCCGCC sequence analogously to the IL-2 sequence in pWS2. The synthetic GM-CSF gene was cloned into the vector pWS2 using the "shot-gun" method (Sambrook, 1989) and sequenced. The errors in the sequence showed up by sequence analysis were corrected by controlled mutagenesis using the phosphorothioate method (Amersham kit).

vi) DNA plasmid construct pGShIL-2tet, containing the sequence coding for human IL-2

An IL-2 cassette, containing the CMV enhancer/promoter, the sequence coding for IL-2 and the SV40-polyA sequence, was obtained by PCR on the basis of the vector pWS2 described in i). The PCR product was subjected to restriction enzyme digestion with EcoRI and cloned into the EcoRI/SmaI site of the plasmid pUC19 (Pharmacia). The plasmid obtained was called pGShIL-2. The source for the tetracyclin resistance gene and parts of the upstream region of the β-lactanase gene (ampicillin resistance gene) was the plasmid pBR327 (Soberon et al., 1980) which had been digested with SspI and AvaI. Together with an EcoRI/AvaI adaptor, the isolated tet sequence was cloned into the EcoRI/SspI site of pGShIL-2. The IL-2 cassette of the resulting clone pGShIL-2tet/amp was sequenced; then the amp sequence was excised with Eam1105I and SspI and the plasmid was re-ligated. The plasmid obtained was designated pGShIL-2tet.

vii) Reporter plasmid construct pCMVL

The plasmid pCMV was prepared by removing the BamHI insert of the plasmid pSTCX556 (Severne et al., 1988), treating the plasmid with Klenow fragment and using the HindIII/SspI and Klenow-treated fragment from the plasmid pRSVL (containing the Photinus pyralis luciferase gene under the control of Rous Sarcoma Virus LTR Enhancer/Promoter (Uchida et al., 1977, De Wet et al., 1987). The reporter plasmid was designated pCMVL.

b) Preparation of transferrin-polylysine conjugates

In order to synthesise conjugates of transferrin and polylysine with a chain length of 290 lysine groups the method described by Wagner et al., 1991b was used.

c) Preparation of EGF-polylysine conjugates

1 mg of EGF (Epidermal Growth Factor, Sigma, St. Louis, Cat. No. E-4127) was purified by gel filtration (Sephadex G-10) using HBS as elution buffer. 135 nmol (0.8 mg) of epidermal growth factor (EGF) in 1.5 ml of HBS were treated with a 15 mM ethanolic solution of SPDP (1.2 µmol). After 2 hours at ambient temperature the modified protein was gel-filtered over a Sephadex G10 column, thereby obtaining 70 nmol of EGF, modified with 50 nmol of dithiopyridine linker. The modified protein was allowed to react with 3-mercaptopropionate-modified polylysine (50 nmol, average chain length 290 lysine monomers, modified with 150 nmol of mercaptopropionate linker) in a total of 1 ml of HBS under an Argon atmosphere. Conjugates were isolated by gel permeation chromatography on a Superdex 75 column (Pharmacia) (buffer: 0.5 M sodium chloride). The product fraction contained a conjugate consisting of 20 nmol of streptavidin and 25 nmol of polylysine.

d) Preparation of streptavidin-polylysine conjugates

The coupling of streptavidin with polylysine was carried out using the method described by Wagner et al., 1990 and in EP-A1 388 758 for the preparation of transferrin-polylysine conjugates.

79 nmol (4.7 mg) of streptavidin in 1 ml of 200 mM HEPES pH 7.9 and 300 mM NaCl were treated with a 15 mM ethanolic solution of SPDP (236 nmol). After 1.5 hours at ambient temperature the modified protein was gel-filtered over a Sephadex G-25 column, thereby obtaining 75 nmol of streptavidin, modified with 196 nmol of dithiopyridine linker. The modified protein was reacted with 3-mercaptopropionate-modified polylysine (75 nmol, average chain length 290 lysine monomers, modified with 190 nmol of mercaptopropionate linker) in 2.6 ml of 100 mM HEPES pH 7.9, 150 mM NaCl under an Argon atmosphere. Conjugates were isolated by cation exchange chromatography on a Mono S HR5 column (Pharmacia). (Gradient: 20 - 100% buffer. Buffer A: 50 mM HEPES pH 7.9; buffer B: buffer A plus 3 M sodium chloride. The product fraction eluted at a salt concentration of between 1.2 M and 1.7 M. Dialysis against HBS (20 mM HEPES pH 7.3, 150 mM NaCl) yielded a conjugate consisting of 45 nmol streptavidin and 53 nmol polylysine.

e) Preparation of biotinylated, inactivated adenovirus

i) Adenovirus dl312 preparations

The adenovirus strain dl312 described by Jones and Shenk, 1979, which has a deletion in the EIa-region was used. Replication of the virus was carried out in the E1a-trans-complementing cell line 293, the preparation being carried out on a large scale as described by

Davidson and Hassell, 1987. The purified virus was taken up in storage buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 0.1% BSA, 50% glycerol) or in HBS/40% glycerol and aliquots were stored at -70°C. The virion concentration was determined by UV-spectrophotometric analysis of the extracted genomic virus DNA (formula: one optical density unit (OD, A_{260}) corresponds to 10^{12} virus particles/ml; (Chardonnet and Dales, 1970)).

ii) Adenovirus dl1014 preparation

The adenovirus dl1014, described by Bridge and Ketner, 1989, was propagated in the cell line W162, which is derived from the cell line Vero (ATCC No. CCL81) and the preparation of which has been described by Weinberg and Ketner, 1983. Large scale production, purification and measurement of the virion concentration were carried out as described for dl312.

iii) Biotinylation of adenovirus

2.4 ml of a gel-filtered (Sephadex G-25 PD10, Pharmacia) solution of adenovirus dl312 (about 10^{11} particles) in 150 mM NaCl/5 mM HEPES, pH 7.9/10% glycerol, was mixed with 10 µl (10 nmol) of a 1 mM solution of NHS-LC-biotin (Pierce 21335). After 3 hours at ambient temperature the biotin-modified virus was separated from the excess reagent by gel filtration (as above). The solution was brought to a glycerol concentration of 40% by the addition of glycerol (total volume 3.2 ml) and stored at -25°C. The biotinylation of the virus could be detected in qualitative measurement after dropwise addition of various dilutions to cellulose nitrite membrane: after drying at 80°C for 2 hours in a vacuum dryer, blocking with BSA, incubating with streptavidin-conjugated alkaline phosphatase (BRL), washing and incubating for 1 hour with the developing solution NBT/X-phosphate

(nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolylphosphate, toluidine salt; Boehringer Mannheim) a positive colour reaction was observed.

In the biotinylation of adenovirus dl1014, the starting material used was 15 ml of adenovirus preparation (1.2×10^{12} particles per ml) which was treated with 150 μl, 1 mM of NHS-LC-biotin. After 3 hours at ambient temperature the mixture was dialysed against 1 litre of HBS plus 40% glycerol overnight at 4°C, then the buffer was replaced with fresh buffer and second dialysis was carried out. A virus preparation was obtained having a titre of about 1.2×10^{12} particles per ml.

iv) Inactivation of biotinylated adenovirus with 8-methoxypsoralen

200 μl of biotinylated virus preparation were placed in each of two wells in a 1.6 cm tissue culture dish. 2 μl (33 mg/ml) of 8-methoxypsoralen (Sigma, Catalogue No. M-3501, dissolved in DMSO) were added to each sample, the dish was placed on ice and irradiated for 10 minutes with a UV lamp (365 nm; UVP TL-33 lamp), the sample being at a distance of 4 cm from the filter. After the irradiation the two samples were combined and gel-filtered (G50, Nick column, Pharmacia), the column previously having been equilibrated with 40% glycerol in HBS.

The biotinylated adenovirus dl1014 was inactivated by combining 4 ml of virus preparation (1×10^{12} virus particles) with 40 μl of 8-methoxypsoralen (33 μg/μl in DMSO). The samples were left to stand for 1 hour at ambient temperature and placed in 12 x 300 μl culture dishes, set on ice and UV-irradiated for 25 minutes as described above. Then gel filtration was carried out (in two batches) over a G-25 PD10 column, using HBS/40%

glycerol. 4.5 ml of virus preparation were obtained with a titre of 9.4×10^{11} particles per ml.

f) Preparation of transfection complexes

The ternary complexes were prepared in three stages. In the first stage, biotinylated adenovirus in 100 µl of HBS was mixed streptavidinylated polylysine (StreptpL) in 100 µl of HBS and incubated for 30 minutes at ambient temperature. Then 6 µg of plasmid DNA in 150 µl of HBS were added, mixed thoroughly and incubation was continued for a further 30 minutes. Finally, polylysine-modified human transferrin (TfpL) in 150 µl of HBS was added, mixed thoroughly and incubated for 30 minutes. (The amounts for adenovirus, StreptpL and TfpL are specified in the individual Examples.) The ratio of plasmid DNA to polylysine conjugates was calculated with respect of electroneutrality in the final complexes. For the transfections the complexes were applied to the cells in a total volume of 2 ml of culture medium without FCS. After 4 hours' incubation at 37°C the medium was removed and fresh culture medium containing serum was added.

g) Cells and culture media

i) Murine melanoma cells

The murine melanoma cell line Cloudman S91 (clone M3) was acquired from ATCC (No. CCL 53.1). The cells were cultured in 6 cm plastic dishes or T25 culture dishes, coated with 0.1% gelatine, in Ham's F10 medium, containing 12.5% equine serum, 2.5% FCS, 2 mM glutamine and antibiotics.

ii) Murine fibroblasts

Primary murine fibroblasts were isolated as follows from DBA/2 mice. DBA/2 mice 4 weeks old were killed by cervical dislocation and dipped in 70% ethanol. The muscles were removed from rear limbs under sterile conditions and washed with PBS. The samples were divided into pieces smaller than 2 mm and excess PBS was removed. Then the tissue fragments were washed three times with 5 ml PBS containing 0.25% trypsin (from pigs' pancreas, Sigma, Catalogue No. T-2904), 0.1% collagenase (type XI, Sigma, Catalogue No. C-9407), 0.1% BSA (fraction V, Boehringer Mannheim, Catalogue No. 735 094) and incubated for 30 minutes at 37°C, with tilting. Then the tissue fragments were left to settle for 5 minutes and the supernatant containing the freed cells was mixed with 5 ml DMEM containing 20% FCS. The non-dissociated material was digested for another 30 minutes and the supernatant was collected as described above. This procedure was repeated until all the material had been dissociated. The combined supernatants were centrifuged for 15 minutes at 500 x g, the cell pellet was resuspended in DMEM containing 20% FCS and the cells were seeded in culture dishes. After 30 minutes the non-adhering cells were suction filtered and fresh medium was added.

iii) Human melanoma cells

Primary human melanoma cells were isolated from surgically removed melanomas. The tumours were ground into small fragments mechanically (using forceps and surgical blade) in the presence of RPMI 1640 culture medium containing 5% FCS, 2 mM glutamine and antibiotics. Then the tissue fragments were carefully pressed through a metal screen using the plunger of a syringe. The material was then washed several times by

centrifuging and resuspension and the cells released were seeded in T25 culture dishes.

iv) Human fibroblasts

After surgical removal, skin biopsies were placed in 4°C DMEM containing 10% FCS, 2 mM glutamine and antibiotics (penicillin/streptamycin or, in the case of human fibroblasts, gentamycin). The biopsies were carefully cut up using forceps and a surgical blade in a tissue culture device in a laminar air current in sterile 6 cm plastic dishes. Then 3 ml of DMEM containing 20% FCS, 2 mM glutamine and antibiotics was added and the culture was placed in a 37°C incubator. After 10 days the medium was replaced by DMEM containing 10% FCS. Then the medium was changed a further two times a week. 4 weeks after the start of the culture, the cells which had grown out of the tissue fragments were trypsinised and plated out into new culture dishes for transfection.

An alternative preferred method consisted in transferring the pieces of skin into fresh medium after they were cut up and washing them with medium once or twice as required. 5 to 10 tissue fragments were placed in a T25 tissue culture dish the surface of which had been wetted with DMEM plus 10% FCS, and uniformly distributed, after which the dish was rotated. This caused the biopsies to hang down ("hanging drop configuration"; this method was described by Jones, 1989). After 1 to 3 hours in the incubator the dishes were rotated again and filled with 1 to 2 ml of medium. Any fixed biopsies were topped up to 5 ml after 24 hours; otherwise the procedure was repeated. After 6 to 10 days the first fibroblasts grew out and from this time on the medium was changed once a week. As soon as the cells were confluent they were transferred into a T75 dish.

v) KB cells

The human epidermoid carcinoma cell line KB was obtained from ATCC (No. CCL 17). The cells were cultured in 6 cm plastic dishes in MEM, 10% FCS, 2 mM glutamine and antibiotics.

h) Determining the gene expression

i) Luciferase assay

The preparation of cell extracts, the standardisation of the protein content and the measurement of the luciferase activity were carried out as described by Zenke et al., 1990, Cotten et al., 1990, in EP 388 758.

ii) IL-2 assay

The expression of interleukin-2 was determined using a bioassay as described by Karasuyama and Melchers, 1988. In addition the IL-2 production was carried out using the IL-2 ELISA kit of Becton Dickinson (Catalogue No. 30032) following the manufacturer's instructions.

iii) IFN-γ assay

The expression of murine-IFN-γ was carried out using the IFN-γ ELISA "Intertest-γ" (Genzyme, Catalogue No. 1557-00).

iv) GM-CSF assay

The expression values for GM-CSF were determined using a commercially obtainable ELISA (Endogen).

j) Testing of complex components

In order to test the predictive qualities of experiments regarding the internalising qualities of ligands or the efficiency of DNA constructs, transfection tests with different virus preparations were carried out in preliminary trials. These trials showed that the results obtained with dl312 can be transferred to psoralen/UV-inactivated adenovirus dl312 and to (psoralen/UV-inactivated) adenovirus dl1014. Therefore, in some of the trials, dl312 was used as a representative.

Example 1

Gene transport to primary human melanoma cell cultures

Primary human melanoma cell isolates obtained from two different patients (HMM-1 and HMM-2) were transfected at various intervals of time after the start of the culture with complexes which contained the following components: 1.7×10^{10} adenovirus particles dl312, 100 ng of StreptpL, 6 µg of pCMVL-DNA, 7 µg of TfpL. 24 hours after transfection the luciferase expression was determined. The expression was standardised on the basis of the protein content of the cell lysates at 1×10^6 cells. The results of these transfection tests are shown in Fig. 1.

Example 2

Determining the ligands involved in the uptake of gene transfer complexes

Primary human melanoma cell isolates designated HMM-1

were transfected with 1.7 x 10^{10} adenovirus particles dl312, 100 ng of StreptpL, 6 µg of pCMVL-DNA and 7 µg of TfpL 2 weeks after the start of the culture. In parallel, the transfection was carried out in the presence of a 50 molar excess of free ice-charged transferrin as a competitor for the internalisation of the TfpL DNA adenovirus complexes. As an alternative, transfection complexes were prepared in which the TfpL was replaced by non-conjugated pL (pL). The results of these tests are shown in Fig. 2. It was found that the addition of free transferrin reduces the amount of luciferase expression, which indicates that the complexes, on the one hand, are at least partially taken up by binding to the transferrin receptor. On the other hand, however, the even more considerable gene expression which is seen in the presence of free transferrin indicates that binding to the adenovirus receptors also makes a major contribution.

Example 3

Influence of the inactivation of adenovirus dl312 with psoralen/UV on long-term toxicity in transfected primary human melanoma cells

3 x 10^5 HMM-1 primary human melanoma cells per 6 cm petri dish were transfected with complexes which consisted of 1.2 x 10^{10} adenovirus particles dl312, 1,200 ng StreptpL, 6 µg pCMVL and 6.6 µg TfpL or 7.5 x 10^9 8-methoxypsoralen/UV-inactivated adenovirus particles dl312 (dl312 PI), 600 ng StreptpL, 6 µg pCMVL and 6.6 µg TfpL (the optimum amount of StreptpL for the various viruses had been determined in preliminary trials by titration). 1 day, 3 days and 7 days after transfection the luciferase expression per 3 x 10^5 cells was measured. The effect of the 8-methoxypsoralen/UV inactivation on

gene expression is shown in Fig. 3. 7 days after the transfection there was a sharp reduction in gene expression which also correlated with serious cytopathic changes in this culture. The transfection of the cells with 8-methoxypsoralen/UV-inactivated adenovirus significantly reduced the cytopathic changes and on the 7th day led to expression values which were 10 times higher than the non-inactivated virus.

Example 4

Determining the long-term cytotoxicity of adenoviruses

Fibroblasts originating from a malignant melanoma were transfected with combined complexes which contained either adenovirus dl312, 8-methoxypsoralen/UV-inactivated adenovirus dl312 or adenovirus dl1014.

The cells were transfected with complexes containing the following components: 6 μg pCMVL-DNA, 0.8 μg StreptpL, 6.8 μg TfpL and 20 μl adenovirus dl312 preparation (0.25×10^{12} virus particles per ml) or 40 μl of 8-methoxypsoralen/UV-inactivated adenovirus dl312 preparation (0.13×10^{12} virus particles per ml) or 3 μl adenovirus dl1014 preparation (4.1×10^{12} virus particles per ml). 100 μl aliquots of this mixture or, in order to obtain the virus:cell ratios specified in Fig. 4, a 1:3 serial dilution of this mixture were applied to 3×10^4 cells in each well of a cell culture plate (24 wells per plate) in 500 μl of RPMI + 2% FCS. After 2 hours at 37°C the medium was replaced by 2 ml of RPMI + 10% FCS. After 8 days the medium was removed and the cells were fixed for 5 minutes with 4% formaldehyde and 150 mM NaCl and then stained with 0.1% crystal violet in 2% ethanol for 10 minutes. The cells were then washed twice with PBS and once with water and photographed.

The results of the cytotoxicity tests are shown in Fig. 4: with a standard ratio of virus to cells of 10,000:1 adenovirus dl312 is cytotoxic; this cytotoxicity is weakened by psoralen/UV treatment of the virus. The same quantity of adenovirus dl1014 showed no cytotoxic effect on the cells.

Example 5

Effect of irradiating the tumour cells on the gene expression

3×10^5 primary human melanoma cells designated HMM-5 or the mouse melanoma cell line M-3 were treated with complexes containing 3×10^9 8-methoxypsoralen/UV-inactivated adenovirus particles dl1014, 600 ng of StreptpL, 6 µg of pCMVL and 6.8 µg of TfpL. 6 hours after transfection the cells were irradiated with doses ranging from 0 - 100 Gy. 48 hours after irradiation the expression of the transfected luciferase gene was measured. The values given in Fig. 5 show the luciferase expression in light units per µg of protein in the cell lysate.

Example 6

Influence of the plasmid concentration on gene expression

3×10^5 M-3 mouse melanoma cells were transfected with complexes consisting of 8.6×10^9 8-methoxypsoralen/UV-inactivated adenovirus particles dl1014, 400 ng StreptpL, 6 µg DNA and 5.1 µg TfpL. The complexes were transfected with plasmid combinations (pCMVL/pSP = pSP65 Boehringer Mannheim; pSVEmu-IFN-γ; IFN-γ/pBCMGneo-mIL-2)

with various proportions of the plasmids. The proportions of the plasmids and the gene expression values obtained are shown in Table I and in Fig. 6 (luciferase expression) and Fig. 7 (IFN-γ/IL-2).

Example 7

Loss of the tumorigenicity of transfected mouse melanoma cells

M-3 mouse melanoma cells (3×10^5 cells per 6 cm petri dish) were transfected with 2×10^9 adenovirus particles dl312, 250 ng of StreptpL, 6 µg of plasmid DNA (containing the mouse IL-2 or the mouse IFN-γ sequence) and 7.5 µg of TfpL. 4 hours after transfection the cells were washed twice with Ham's F10 culture medium without serum. Then 1×10^5 cells were subcutaneously administered into the backs of 6 anaesthetised DBA/2 mice. As a control, another group of DBA/2 mice were given 1×10^5 M-3 cells which had not been transfected. The tumour growth at the injection site was monitored weekly. Fig. 8 shows the course of tumour formation.

Example 8

Induction of a systemic immune response against melanoma by immunising with cytokine-transfected, irradiated tumour cells ("prophylactic mouse model")

a) M-3 melanoma cells (3×10^5 cells per T25 culture dish) were transfected with 3×10^9 8-methoxypsoralen/UV activated adenovirus particles dl1014, 6 ng of StreptpL, 6 µg of IL-2 plasmid DNA or pSP plasmid DNA which contains no cDNA insert and therefore does not lead to expression of a gene product in the transfected cells,

and 6.8 µg of TfpL. 4 hours after transfection the transfection complexes were removed and fresh medium containing serum was added. Then the cells were irradiated 6 hours after transfection with X-rays at a dose of 20 Gy and cultivated for a further 18 hours. 24 hours after transfection the cells were trypsinised and washed twice with Earl's buffered saline solution (EBSS) and the culture was adjusted to a concentration of 1×10^6 per ml. Anaesthetised DBA/2 mice were immunised with 1×10^5 cells administered subcutaneously into the back. In parallel, a group of 6 mice were immunised with non-transfected M-3 cells which had been irradiated and further treated in the same way as the transfected cells. One week after the first immunisations the mice were given booster immunisations with cell preparations as used for the first immunisations. After another week the animals were exposed to the highly tumorigenic dose of 1×10^5 M-3 cells administered at a site which was remote from the earlier immunisation sites. In addition, 4 mice which had not been immunised were exposed to the tumorigenic cells in the same way. 8 weeks after the implantation of the tumour cells all (4/4) non-immunised animals had developed tumours, whereas all the mice which had been immunised with the irradiated IL-2 transfected M-3 cells were free from tumours (0 tumours in 5 animals). The mice which had been treated with irradiated, non-transfected M-3 cells and with irradiated M-3 cells which had been transfected with an "empty" plasmid (pSP) were only partly protected (4 out of 6 mice developed tumours). The development of the tumours in the animals is illustrated in Table II and in Fig. 9. The results obtained show that the immunisation of mice with IL-2 transfected, irradiated tumour cells induces a systemic immune response which protects the animals from further tumour development.

b) In another test series the mice were treated, in

accordance with the same immunisation programme, with irradiated M-3 cells which had been transfected with complexes containing 3 x 10^9 8-methoxypsoralen/UV-inactivated adenovirus particles dl1014, 600 ng of StreptpL, a) 6 µg of IL-2 plasmid pBCMGneo-mIL-2 (IL-2 100%), b) 5.4 µg of pSP plasmid and 0.6 µg of IL-2 plasmid (IL-2 10%), c) 5.4 µg of IL-2 plasmid and 0.6 µg of IFN-γ plasmid (IL-2 90% + IFN-γ 10%) or d) 5.4 µg pSP plasmid and 0.6 µg IFN-γ plasmid (IFN-γ 10%), and 4.7 µg TfpL. In addition, a group of mice were immunised with irradiated M-3 cells which had been transfected with 3.6 x 10^9 inactivated adenovirus particles dl312, 600 ng StreptpL, 6 µg IL-2 plasmid and 4.7 µg TfpL (IL-2 100% dl312). One week after the booster injection, 1 x 10^5 M-3 cells were implanted in the immunised animals and, as a control, in the non-immunised animals as well. The development of the tumours is summarised in Table III.

c) Using the same immunisation programme as in the previous Examples, the mice were treated with irradiated M-3 cells which had been transfected with complexes containing 3 x 10^9 8-methoxypsoralen/UV-inactivated adenovirus particles dl1014, 600 ng of StreptpL, a) 6 µg of IL-2 plasmid = 100% IL-2, b) 5.76 µg pSP plasmid and 0.24 µg IL-2 plasmid = 4% IL-2, or c) 6 µg pSP plasmid and 4.7 µg TfpL. The production of IL-2 by the transfected irradiated cells was measured on the day of immunisation: M-3 cells which had been transfected with 100% IL-2 plasmid produced 33,000 units of IL-2 per 1 x 10^6 cells in 24 hours, whereas M-3 cells transfected with 4% IL-2 plasmid produced 396 units of IL-2 per 1 x 10^6 cells in 24 hours. One week after the booster injection 1 x 10^5 M-3 cells were implanted in the immunised animals and, as a control, in the non-immunised animals as well. In order to obtain more information as to the degree of the immune response induced by the immunisations using different quantities

of IL-2 plasmid, 3 x 10^5 M-3 cells and 1 x 10^6 M-3 cells were implanted, respectively, in other groups of mice. In order to demonstrate that the immune response is tumour-specific, 1 x 10^5 syngenetic plate epithelial carcinoma cells KLN 205 (ATCC No. CRL 1453) were implanted in a group of mice which had been immunised with 100% IL-2 transfected irradiated M-3 cells. The development of the tumours is shown in Table IV.

Example 9

Gene transfer into cells of an epidermoid carcinoma cell line

KB-cells were cultured in a density of 400,000 cells per 6 cm dish and transfected with various complexes which contained 2.5 x 10^9 adenovirus particles dl312, 200 ng of StreptpL, 6 μg of pCMVL-DNA and, as desired, 3.8 μg of polylysine, 3.8 μ of EGF-pL (quantity based on polylysine content) or 6 μg of TfpL. The complexes (500 μl each) were mixed with 1.5 ml of medium either with or without 2% FCS. In competitive experiments, a further 1 μg of EGF was added to the mixture of the complex and medium. The complexes with medium were added to the cells. After 2 hours' incubation at 37°C the medium was removed and fresh medium containing serum was added.
Harvesting of the cells after 24 hours and luciferase assay produced the following results (Fig. 10A: experiment with 2% FCS, Fig. 10B: experiment without FCS): Fig. 10A: with EGFpL a value of 20845000 light units was obtained; this value was substantially higher than with TfpL (3970000) or polylysine (plys 10550000). In competitive experiments with free EGF, by contrast with the experiments using TfpL (TfpL+EGF 12350000 light units) or polylysine (pLys+EGF 14055000) the expected

reduction in the signal was found (EGFpL+EGF 14150000). This shows that the intensified gene transfer proceeds ligand-specifically via the EGF receptor. In the experiment without FCS (Fig. 10B) the effect of EGFpL was even more marked (EGFpL 15150000, TfpL 2000000, pLys 5100000, EGFpL+EGF 5900000 light units). The expression values relate to 50% of the cells.

Analogously, KB-cells were transfected with complexes which contained 5 x 10^9 8-methoxypsoralen/UV-inactivated adenovirus particles dl1014; comparable luciferase expression values were obtained.

Example 10

IL-2 expression using different vectors

a) M-3 cells

3 x 10^5 M-3 cells were transfected with various plasmid constructs which occurred as components of ternary complexes. 3 µl of adenovirus dl312 preparation, 0.3 µl (about 200 ng) of StreptpL, 6 µg of TfpL and 6 µg of plasmid DNA (BCMGneo-mIL-2, pWS2m, BMGneo-mIL-2 or pCM2) were made up to a total volume of 500 µl using HBS.

For the experiments with 8-methoxypsoralen/UV-inactivated adenovirus dl1014 the following transfection complexes were used: 9 µl of virus preparation, 600 ng of StreptpL, 6 µg of TfpL and 6 µg of BCMGneo-mIL-2.

After the time intervals specified in Fig. 11, the IL-2 activity was determined. For BCMGneo-mIL-2, in addition, 64,000 units were obtained after 48 hours and 7,128 units were obtained after 28 days (not shown in the Figure). For pCM2, 3,227 units were obtained after

48 hours and 950 units after 28 days, for BMGneo-mIL-2 396 units were obtained after 24 hours, 604 units after 48 hours, 297 units are 7 days and 264 units after 14 days (not shown in the Figure).

b) Fibroblasts

Primary human fibroblasts were transfected with IL-2 constructs as in a) and the IL-2 expression was determined on the days after transfection specified in Fig. 12. The composition of the transfection medium was the same for BCMGneo-mIL-2 as for M-3 cells; an identical transfection complex was prepared for BMGneo-mIL-2.

Example 11

Melanoma cells which express HSA on their surface stimulate an immune response with a protective effect against non-modified tumour cells

M3 cells were cultured in Ham's F12 medium plus 12.5% equine serum, 2.5% FCS on gelatine-coated plastic dishes. 24 hours before transfection the cells were plated out in a 25 cm^2 culture flask in amounts of 3×10^5. The transfection complexes for this quantity of cells were prepared by combining 9 μl of biotinylated, 8-methoxypsoralen/UV-inactivated adenovirus dl1014 (1.4×10^{12} particles/ml), 5.2 μg of transferrin polylysine, 800 ng of streptavidin-polylysine and 6 μg of the associated plasmid DNA (pSP, pWS2m combined with pSP, pHSA in conjunction with pSP or IL-2 in conjunction with pHSA) in a total volume of 500 μl. This volume was added to each culture flask in 2 ml of Ham's F12 medium plus 12.5% equine serum/2.5% FCS. After 2 hours' incubation at 37°C the medium was replaced by fresh

medium and the cells were irradiated (20 Gy). 24 hours after transfection the cells were trypsinised, washed twice with HBSS (Hank's Buffered Saline Salts), counted and taken up again in 100 µl of HBSS in quantities of 100,000 cells. These cells formed the vaccine which was used within 60 minutes for injection into the recipient mice.

The mice were injected as described in the previous Examples. Two separate vaccine injections were given each containing 100,000 cells, at two different sites on the back, at an interval of one week. One week after the second injection the mice were injected with 300,000 unmodified, unirradiated M3 cells in 300 µl of HBSS at a third site on the back. The development of the tumours was monitored at intervals of one week.

Fig. 13 shows the results of these experiments (the x axis gives the number of days whilst the y axis gives the size of the tumour in mm^3. The numbers beside the end points indicate the number of mice with tumours compared with the total number of mice in each group. pSP: 6 µg pSP; 80 IL-2/20 pSP: 4.8 µg pWS2m plus 1.2 µg pSP; 20 HSA/80 pSP: 1.2 µg pHSA plus 4.8 µg pSP; 80 IL-2/20 HSA: 4.8 µg pWS2m plus 1.2 µg pHSA): vaccination with irradiated M3 cells transfected with the empty vector pSP did not confer protection against tumour growth; in 4 of the 6 mice considerable tumour masses were formed. The IL-2 producing cells brought about only a moderate regression of the tumours in all the mice in the group (3/3) which developed tumours. (This effect can be ascribed to the fact that the test conditions were selected with regard to testing the advantageous effect of HSA, using mice which were given the triple dose of tumour cells but a reduced dose of IL-2 DNA.) By contrast, the mice which had been vaccinated with HSA-expressing M3 cells showed sharply

suppressed tumour growth. The mice which had received vaccination with both HSA and IL-2 expressing M3 cells developed tumours with greatly reduced frequency (2 out of 6) and the tumours were small compared with the control tumours (vaccination with pSP-transfected cells); the average size was 18 mm^3 compared with 1149 mm^3.

Example 12

Melanoma cells which express the rabies neoantigen on their surface stimulate an immune response with a protective effect against unmodified tumour cells.

M3 cells were treated and transfected as described in Example 11. The only difference was that the duration of effect of the transfection complexes, which were prepared exactly as in Example 11 (6 μg of plasmid DNA), on the cells was four instead of two hours. The injections into the mice were given analogously to Example 11, except that the interval between the injections was extended from one to two weeks and the injection volume was 100 μl in each case.

Fig. 14 shows the results of the tests; the construction of the graph is analogous to that of Fig. 13. The pSP-negative control shows the expected considerable tumour growth. The positive control in which only pWS2m (=100% IL-2) was transfected shows total protection; the transfected cells produced 45,000 IL-2 units/10^6 cells in the 24 hours after transfection and before injection. In the group of mice which were given only 50% pWS2m (with 50% empty vector pSP) incomplete protection was observed. This was significantly improved if instead of the empty vector the rabies expression plasmid pWS-rabies was co-transfected. Slight tumour growth between

weeks 2 and 4 regressed from week 5 onwards.

Example 13

Comparison of various methods of inactivating adenoviruses

Different methods were used within the scope of this Example and were compared in terms of the reduction in the virus titre and the ability of the inactivated viruses to intensify the gene transfer via receptor-mediated endocytosis. Trials were also carried out to see which virus genes are switched off by the inactivation.

a) Testing the inactivation of adenoviruses with 8-methoxypsoralen

i) Inactivation of the viruses

Samples of biotinylated adenovirus dl1014 (300 µl) in HBS/40% glycerol were added to the 4 wells of a cell culture dish (NUNC, Catalogue No. 176740). Aliquots of 33 mg/ml of 8-methoxypsoralen in DMSO were added to the viruses in order to obtain the desired final concentrations. The samples were placed on ice with their lid closed and irradiated for 25 minutes with UV radiation as described in e) iv), the position of the sample plate being changed every 10 minutes to avoid shadows. Unreacted psoralen was removed by gel filtration: in order to do this the virus/psoralen sample (2 ml) was applied to a Pharmacia PD-10-gel filtration column (pre-equilibrated with 30 ml of HBS/40% glycerol). The sample was washed with 0.5 ml HBS/40% glycerol into the column and the virus was eluted with HBS/40% glycerol, the first 400 µl being

discarded and the following 4 ml being collected in fractions of 0.5 ml. In order to identify the virus fractions, a ninhydrin assay was carried out, the positive fractions were combined, the protein concentration was measured and the virus was deep-frozen in aliquots at -70°C.

ii) Titration of 8-methoxypsoralen (CPE against gene transfer)

The titrations were carried out in order to establish the optimum psoralen concentration which does not affect the DNA transporting ability of the virus whilst totally inactivating the virus. (There are reports indicating that a poor inactivation performance is achieved if psoralens are used in concentrations close to saturation, whilst substantially better performances are achieved at lower concentrations. This might be due either to a phenomenon of crystallisation, which removes the compound from the solution, or to a filter effect, the latter because high concentrations of unbound compounds absorb the UV-radiation and thereby block the activation of DNA-bound material by UV.)

The CPE assay ("Cytopathic Effect Assay"; Precious and Russell, 1985) for determining the virus titre was carried out using W162 cells which had been plated out on 24-well plates in a quantity of 50,000 cells/well. The serial dilutions of the samples were carried out in DMEM/2% heat inactivated equine serum and the dilute virus was applied to the cells in 500 µl DMEM/2% heat inactivated equine serum. After two hours incubation at 37°C the medium was replaced by fresh DMEM/10% FCS. After 4 to 5 days at 37°C the cells were fixed with formaldehyde and stained with crystal violet.

The suitability of the method of inactivation method in

terms of gene transfer was tested using the introduction of the luciferase gene into K562 cells (ATCC No. CCL 243). The transfections using combined complexes were carried out as described in WO 93/07283, using 9 μl of adenovirus dl1014 (1.4×10^{12} particles/ml) or by inactivating with β-propiolactone 13.5 μl (9.3×10^{11} particles/ml), 800 ng of streptavidin-polylysine, 6 μg of pCMVL-DNA and 5.2 μg of transferrin-polylysine in 500 μl of HBS (the complexes were prepared as described in the methods under f)). Moreover, the relative titre of each virus preparation was determined by CPE-assay on W162 cells. The results of these titrations are shown in Fig. 15: the numbers on the left-hand side of the Figure relate to the relative virus titre (solid triangles) the numbers on the right-hand side of the Figure relate to luciferase light units (open squares). The x axis gives the concentration of 8-methoxypsoralen in mg/ml. It was found that treatment of the virus with 0.11 mg/ml of 8-methoxypsoralen brings about a reduction in the titre which does not differ from that caused by a concentration of 0.33 mg/ml in preliminary trials. The DNA transporting activity is retained at this concentration. Under the conditions used, an 8-methoxypsoralen concentration of 0.033 mg was ineffective. (More sensitive analysis using plaque assay, see Fig. 19, showed that concentrations of 0.11 and 0.33 mg/ml of 8-methoxypsoralen produced comparable reductions in the virus titre.)

b) Testing the inactivation of adenoviruses with 4'-aminomethyl-4,5',8-trimethylpsoralen

i) Inactivation of the viruses

The less hydrophobic psoralen derivative 4'-aminomethyl-4,5',8-trimethylpsoralen is charged; it interacts with and consequently inactivates single-stranded RNA viruses

as well as DNA viruses and is soluble in aqueous solutions in amounts of 5 mg/ml. The 4'-aminomethyl-4,5',8-trimethylpsoralen used was obtained from H.R.I. (Catalogue No. 6) and dissolved in HBS in amounts of 5 mg/ml. After aliquots thereof had been added to the virus samples, the virus was inactivated and purified in the same way as for 8-methoxypsoralen.

ii) Titration of 4'-aminomethyl-4,5',8-trimethylpsoralen (CPE against gene transfer)

The titrations were carried out exactly as described under a) ii) for 8-methoxypsoralen. The results of these titrations are shown in Fig. 16: the numbers on the left-hand side of the Figure relate to the relative virus titre (solid triangles) whilst the numbers on the right-hand side of the Figure relate to luciferase light units (open squares). The x axis shows the concentration of 4'-aminomethyl-4,5',8-trimethylpsoralen in μg/ml. With 0.3 and 1 mg/ml of 4'-aminomethyl-4,5',8-trimethylpsoralen conditions were established which bring about a fall in titre of at least 5 log, whilst the efficiency of DNA transfer is maintained. Lower concentrations of 4'-aminomethyl-4,5',8-trimethylpsoralen (<0.1 mg/ml) bring about only a moderate fall in the virus titre. The plaque assay (see Fig. 19) also showed that concentrations of about 1.0 and 0.3 mg/ml of 4'-aminomethyl-4,5',8-trimethylpsoralen brought about reductions in the virus titre which could not be distinguished from those achieved with 0.11 and 0.33 mg/ml of 8-methoxypsoralen. The gene transfer capacity of the inactivated virus was determined as described under a) ii). In addition, the relative titre of each virus preparation was determined by CPE-assay on W162 cells.

c) Testing the inactivation of adenoviruses with β-propiolactone

i) Inactivation of the viruses

The virus samples were placed on 0.3 M HEPES, pH 7.9 before the addition of the 10-fold concentrated β-propiolactone solutions. Concentrated β-propiolactone solutions were prepared by diluting β-propiolactone (Sigma, Catalogue No. P5648) with HBS immediately before use. Control trials were carried out to show that 0.3 M HEPES, pH 7.9, was sufficient to buffer the β-propiolactone treatment at 1%. Aliquots of β-propiolactone were added to the buffered virus solutions at ambient temperature, then the virus samples were incubated for 4 hours at ambient temperature, before either being stored at -70°C or used for the transfection experiments.

ii) Titration of β-propiolactone (CPE against gene transfer)

The adenoviruses were exposed to various concentrations of β-propiolactone for 4 hours at ambient temperature. Otherwise, the experiments were carried out as described for the psoralen derivatives. It was found (Fig. 17) that treatment with 0.3% β-propiolactone causes a fall in the virus titre of nearly 5 log, whilst the DNA transporting activity is unimpaired. Concentrations of 1% and above bring about an even sharper fall in titre, but the DNA transporting is also impaired. (The numbers on the left in the Figure relate to the relative virus titre (solid triangles), whilst the numbers on the right of the Figure relate to luciferase light units (open squares). The x axis shows the concentration of β-propiolactone in %.)

iii) Gene transfer capacity of the adenovirus after several β-propiolactone treatments are low concentration

The sharp fall in DNA transfer activity observed in ii) with concentrations of between 0.3% and 1% β-propiolactone leads one to assume that, at the lower concentration, a modification occurs preferably to viral nucleic acids, whilst the agent at the higher concentrations begins to modify the capsid proteins and damage the endosomolytic activity of the virus. To test the accuracy of this supposition, the adenoviruses were treated with several aliquots of lower (<0.3%) β-propiolactone concentrations, in the hope of thereby bringing about modification of the viral DNA without damaging the virus proteins. For this purpose, virus samples were treated with one addition of 0.3% β-propiolactone, two additions of 0.3% β-propiolactone, three additions of 0.2% β-propiolactone, four addition of 0.15% β-propiolactone or one addition of 1% β-propiolactone. The gene transfer activity of these virus preparations, which were incorporated in combined complexes as described under f) was tested with K562 cells as described above. As shown in Fig. 18, apart from the 1% sample all treatments caused a drop in the gene transfer capacity of less than 1 log. The treatment with 1% β-propiolactone caused a drop of more than 2 log. (Sample 1: non-inactivated virus. Sample 2: inactivation with 0.11 mg/ml of 8-methoxypsoralen. Sample 3: inactivation with 0.3% β-propiolactone. Sample 4: inactivation with 2 x 0.3% β-propiolactone. Sample 5: inactivation with 3 x 0.2% β-propiolactone. Sample 6: inactivation with 4 x 0.15% β-propiolactone. Sample 7: inactivation with 1% β-propiolactone. The numbers appearing by the bars indicate luciferase light units.)

d) Determining the replication qualities of inactivated adenoviruses by plaque-assay

i) Comparison of the plaque-assays of adenovirus dl1014 inactivated with 8-methoxypsoralen, β-propiolactone or 4'-aminomethyl-4,5',8-trimethylpsoralen

The plaque-assay provides a more sensitive measurement of the replication qualities of the virus: adenovirus 5 requires the penetration of 10 to 50 virus particles in order to produce an infected cell. The CPE-assay shown hereinafter measures the ability of chemically inactivated viruses to trigger a cytopathic virus infection, but this requires the infection of at least 10% of the target population in order to be capable of detection during the four days during which the assay takes. This assay thus makes it possible to detect 50,000 virus particles. By contrast, under optimum conditions, using the plaque assay a single plaque can be detected which is produced as a result of the penetration of 10 to 50 viruses; the test is therefore about 1,000 times more sensitive than the CPE-assay.

The following method was used for the plaque assays carried out within the scope of the present invention: W126 cells were plated out 18 hours before the start of the assay in amounts of 500,000 cells per well in a 6-well plate. Then the medium was removed and replaced by fresh medium (2% equine serum/DMEM) plus the appropriate virus dilution, the virus being uniformly distributed over all the cells. The plates were then incubated for 1.5 hours at 37°C, the plate being carefully tilted every 20 minutes. In the meantime a 2X agarose solution (2% (2 g/100 ml) of SeaPlaque-Agarose of a low gelling temperature (FMC Catalogue No. 5010) in 5 mM HEPES pH 7.4, pH-adjusted before the 25 minute autoclaving) was heated to 70°C in order to melt the agarose and then

equilibrate it at 37°C. A 2X DMEM/10% FCS solution was prepared in a similar manner (double concentration DMEM/double concentration penicillin/streptomycin/double concentration glutamine/10% FCS (heat inactivated at 56°C, 30 min)). At the end of the 1.5 hour incubation of the cells with the virus a 50 ml batch of a coating was prepared (25 ml 2X agarose + 25 ml 2X DMEM/10% FCS in a 50 ml Falcon tube). The medium/virus solution was removed from a plate and 3.5 ml of coating were placed in each well. The plates were then left to stand for at least 30 minutes undisturbed at room temperature in order to harden the agarose before they were incubated at 37°C again. On the 6th day after addition of the virus each well was coated with another 2-3 ml of the coating solution. Using this procedure the plaques are normally visible on the 7th to 10th day. The plaques were counted on the 14th to 18th days after infection.

The testing of various inactivated virus preparations by plaque assay showed that β-propiolactone treatment (2 x 0.3%) brings about a fall of about 5 log in the virus titre (Fig. 19: Sample 1: non-activated virus, Sample 2: inactivation with 0.33 µg/ml of 8-methoxypsoralen, Sample 3: inactivation with 0.11 µg/ml of 8-methoxypsoralen, Sample 4: inactivation with 2 x 0.3% β-propiolactone, Sample 5: inactivation with 0.28 mg/ml 4'-aminomethyl-4,5',8-trimethylpsoralen, Sample 6: inactivation with 0.83 mg/ml of 4'-aminomethyl-4,5',8-trimethylpsoralen. The numbers given at the bars indicate pfu's/ml). No plaques were observed either in the 8-methoxypsoralen-treated (0.33 or 0.11 mg/ml) or in the 4'-aminomethyl-4,5',8-trimethylpsoralen treated (0.28 or 0.83 mg/ml) adenoviruses. With a dilution factor of 6 log between the non-inactivated dl1014 (with 1 x 10^8 pfu/ml) and the psoralen-inactivated samples, this shows that in the psoralen-inactivated samples there are less than 10^2

plaque-forming units (pfu's) present. The essential observation is that β-propiolactone-inactivated viruses form plaques with detectable frequency. Thus, even though the β-propiolactone inactivation in the CPE-assay appears to be equivalent to psoralen inactivation, the plaque assay shows a significant difference between the two types of compounds.

ii) Comparison of the plaque assays of adenovirus dl1014, inactivated with 8-methoxypsoralen or various β-propiolactone treatments

The adenovirus dl1014 preparations were inactivated with various multiple additions of β-propiolactone (see Fig. 20), analysed by plaque assay and compared with adenoviruses which had been inactivated with 0.11 mg/ml of 8-methoxypsoralen. (Sample 1: non-activated virus, Sample 2: inactivation with 0.11 μg/ml of 8-methoxypsoralen, Sample 3: inactivation with 0.3% β-propiolactone, Sample 4: inactivation with 2 x 0.3% β-propiolactone, Sample 5: inactivation with 3 x 0.2% β-propiolactone, Sample 6: inactivation with 4 x 0.15% β-propiolactone, Sample 7: inactivation with 1% β-propiolactone. The numbers at the bars indicate pfu's/ml.) This test, illustrated in Figure 20, also provided no evidence of viruses in the psoralen-treated sample. The dilution factor of 7 log between non-inactivated dl1014 of 6.7×10^8 pfu/ml and the psoralen-inactivated virus shows that there must be fewer than 6.7×10^1 pfu's present in the psoralen-inactivated sample. By contrast, plaques were detected in all the β-propiolactone-treated samples, corresponding to an inactivation of about 2 log (0.3% β-propiolactone) to 5 log (2 x 0.3% β-propiolactone). Although 1% β-propiolactone led to a sharp fall in the DNA transporting activity (cf. Fig. 17), it led to only a moderate fall in the virus titre, which backs up the

assumption that the higher β-propiolactone concentration prefers the protein modification to the DNA modification and therefore has a greater effect on the ability of the virus to break open the endosomes (and hence on the entry of virus) than the virus replication.

e) Gene expression of inactivated virus

Various combined complexes were prepared corresponding to the method described under f), containing optimum amounts of adenovirus dl312, 8-methoxypsoralen inactivated dl312, adenovirus dl1014, 8-methoxypsoralen inactivated dl1014 and β-propiolactone-inactivated dl1014. The complexes contained about 1 x 10^{10} virus particles, 800 ng of streptavidin polylysine, 6 μg of pCMV-DNA and 5.2 μg of transferrin polylysine in a final volume of 500 μl HBS. The complexes were applied to K562 cells (cultivated for 24 hours beforehand in 50 μM deferrioxamine/RPMI/10% FCS, plated out in quantities of 250,000 cells/ml) at the rate of 2 ml/well in a 24-well plate. After 2 hours' incubation the cells were washed in fresh medium without deferrioxamine and 48 hours later they were harvested in order to determine the luciferase activity or subjected to RNA analysis for selected adenovirus genes, using the combined material from three separate transfections.

The measurements of the luciferase activity showed that all the transfections yielded an expression within one order of magnitude.

i) Northern analysis

The RNA northern analysis was carried out using the method described by Paeratakul et al., 1988: 48 hours after transfection the cells were washed three times in HBS and serial dilutions corresponding to 30,000, 10,000

or 3,000 cells were applied to a nitrocellulose filter using a 96 sample dot blot apparatus (Schleicher & Schuell). Then the cells were fixed with 1% glutaraldehyde in HBS for 60 minutes at 4°C, followed by proteinase K digestion (20 µg/ml) and 30 minutes in HBS/0.1% SDS at 37°C. The filter was then pre-hybridised in Church buffer (0.5 M sodium phosphate, 7% SDS, 1 mM EDTA pH 8) at 65°C for 5 hours, followed by incubation (overnight at 65°C) with the labelled DNA probes in a minimum volume of Church buffer. The filters were then washed twice in 2X SSC/0.1% SDS for 30 minutes at 65°C, followed each time by 30 minutes in 0.1X SSC, 0.1% SDS. The radioactive pattern was made visible by phosphorimaging. The radioactively labelled (^{32}P) probes were prepared from PCR-products of adenovirus dl1014 sequences. An E1a-probe (383 bp) was prepared using PCR-primers to Ad5, bp 736 - 751 (E1a.1) and bp 1119 - 1101 (E1a.2). (This sequence is part of the region which is deleted in dl312. It acts as a control; the RNA signal should be missing from the dl312 samples but present in dl1014 samples, as the latter virus has a Wild-type E1-region.) An E3 probe (436 bp) was prepared by the most strongly expressed E3 gene, the 19 K glycoprotein gene (Gooding, 1992) using primers at Ad5, bp 28722 - 28737 (E3.a) and 29157 - 29142 (E3.b) (the expression of E3 would appear to play an important part in the immune response to transfected cells, because at least two of the E3 genes modulate the surface expression of MHC class I molecules and TNF-receptor-molecules on the surface of infected cells.) After the PCR the reaction products were purified on low-melting agarose gels in TAE-buffer, the desired DNA fragment was excised from the gel, weighed and taken up in 1.3 ml of water/g of gel fragment. The gel fragment taken up in water was then heated to 95°C for 10 minutes and 10 µl of the resulting solution were radiolabelled for 12 hours at ambient temperature using the Random Primer Method

(Feinberg and Vogelstein, 1984). Then the labelled DNA was purified by ethanol precipitation in the presence of ammonium acetate and tRNA.

Fig. 21 shows the autoradiogram: it was found that, in the dl312 samples, both the non-inactivated and the 8-methoxypsoralen-inactivated ones, there was no E1a-expression. The expression of E1a is strong in the non-inactivated dl1014 viruses but completely absent both from the 8-methoxypsoralen-treated viruses and from the viruses inactivated twice with 0.3% β-propiolactone. E3 is less strongly expressed in non-inactivated dl312 adenoviruses than in dl1014. This finding accords with the knowledge of the function of E1a as a positive modulator of E3 expression and the expression of other early genes (Nevins, 1991 and 1992). In every case, the inactivation with 8-methoxypsoralen or with β-propiolactone blocked the RNA synthesis by these genes completely.

ii) Reverse Transcription-PCR-Analysis

The cells used as starting material were those which had been transfected as described in the previous Examples, using either non-activated adenovirus dl1014 or 8-methoxypsoralen-inactivated dl1014. In both transfections, identical quantities of virus were used. The RNA purification was carried out with 1 x 10^6 transfected M3 cells, harvested 24 hours after transfection by adding to PBS and brief pelleting in an Eppendorf centrifuge. The cell pellets were dissolved in 1 ml of Trisolv (= phenol/guanidine thiocyanate in a monophase solution; Biotecx) and briefly mixed in a vortex. Chloroform was then added (500 µl), after which two phases were formed of which the aqueous one contained the cellular RNA. The RNA was precipitated from the aqueous phase by the addition of an equal

volume of isopropanol, the precipitate was collected by centrifuging, the cell pellet was washed once with 80% ethanol and dissolved in 20 µl of RNase-free water. Contaminating DNA contained in the RNA sample was eliminated by digestion with RNase-free DNase (Boehringer Mannheim; 60 minutes at 37°C). The DNase was then inactivated by further incubation at 95°C for 5 minutes.

The reverse transcription reaction mixtures contained 10 µl of RNA solution, 2 µl of 10 mM dNTPs, 4 µl of 25 mM $MgCl_2$, 2 µl of 10 X RT buffer (100 mM Tris-HCl, 900 mM KCl, pH 8.3), 1 µl 50 µM oligo $d(D)_{16}$, 1 µl 20 units/µl RNase-inhibitor (Perkin Elmer) and 1 µl MuLV Reverse Transcriptase (Perkin Elmer). Reverse Transcriptase reactions were carried out for 10 minutes at 25°C and for 15 minutes 42°C, followed by 5 minutes at 95°C, in order to inactivate the Reverse Transcriptase.

Primers were used which corresponded to the following sections of the adenovirus 5 genome: E1a up: bp 1228-1248, E1a down: bp 1545-1526, E3 up: bp 28722-28737, E3 down: 29157-29140.

The polymerase chain reaction mixtures contained 35 µl of water, 5 µl of PCR buffer (100 mM Tris-HCl, pH 8.9, 1 M KCl, 15 mM $MgCl_2$), 1 µl 10 mM dNTPs, 1 µl up and down primer (25 mM), 0.5 µl Taq polymerase (5 units/µl, Boehringer Mannheim) and 6.5 µl of the dilute cDNA samples (RNA as control reaction). The samples were denatured for 90 seconds at 95°C, followed by 35 cycles of 30 seconds at 95°C, 60 seconds at 60°C, 30 seconds at 72°C with a final extension of 3 minutes at 72°C. The amplified DNA products were separated on a 2% agarose TAE gel and made visible by staining with ethidium bromide. Fig. 22 shows that, in the non-inactivated adenovirus, the expected PCR-products are visible from

both the E1a- and the E3-region, the signal being detectable at a dilution of 1:1,000 of the target nucleic acid (the upper tables in the Figure). By contrast, no signal could be detected in the samples which originated from the psoralen-inactivated virus. The absence of the PCR signal in a control sample in which the Reverse Transcriptase reaction had been omitted confirms that the signals observed can be put down to the amplification of the RNA and do not come from contamination of the adenovirus-DNA in the nucleic acid preparation.

f) Analysis of the binding of 8-methoxypsoralen to the adenovirus genome

i) Quantitative measurement of 8-methoxypsoralen bound to the adenovirus genome

0.41 ml of tritium-labelled 8-methoxypsoralen (0.8 mCi/ml) were dried, dissolved in 20 µl of DMSO and mixed with 8.7 µl of unlabelled 8-methoxypsoralen (33 µg/µl in DMSO). This mixture was added to 1.8 ml of biotinylated adenovirus dl1014. UV-irradiation and purification by PD-10 chromatography were carried out as described above. The incorporation of ^{3}H 8-methoxypsoralen was determined by counting aliquots of the purified virus in scintillation liquid. Calculations based on the radioactivity measured which was incorporated in the DNA showed that there was one psoralen molecule present to every 800 base pairs of the virus. Additionally, it was found by thin layer chromatographic analysis (silica gel, dichloromethane as solvent) that no free unreacted 8-methoxypsoralen remained in the sample.

ii) Investigation of the location of 8-methoxypsoralen deposits in the virus genome

In order to determine whether the deposits of 8-methoxypsoralen are distributed through the entire virus genome or concentrated at certain accessible sites in the viral DNA, the viral DNA was purified and cleaved by means of restriction enzymes in order to obtain ten or eleven DNA fragments: from the ^{3}H 8-methoxypsoralen-labelled virus the DNA was purified by incubating the virus for 45 minutes at 56°C with 0.4% SDS/0.4 mg/ml proteinase K. The sample was extracted twice with phenol/chloroform (1:1) and once with chloroform, the DNA was precipitated from the aqueous phase by the addition of 1/10 volume 3M sodium acetate, pH 5, with 0.54 volumes of isopropanol. The precipitated DNA was centrifuged, washed twice with ice-cold 80% ethanol, dried in the air and dissolved in TE. DNA aliquots of non-inactivated adenovirus or of ^{3}H 8-methoxypsoralen-labelled adenovirus were digested with the restriction enzymes HindIII or Asp718 in according with the manufacturer's instructions (Boehringer Mannheim), but using 10 times higher amounts of enzyme, purified by extraction with phenol/chloroform and chloroform, precipitated with ethanol and separated on 0.9% agarose/TAE gel in the presence of ethidium bromide (Sambrook et al., 1991). The gel was dried, impregnated with fluorescent scintillant (Enhance, Amersham) and brought into contact with an X-ray film. Fig. 23 shows the gel stained with ethidium bromide and the fluorogram; the labelling pattern shows that ^{3}H 8-methoxypsoralen is distributed through the genome. With a few exceptions, the fragments obtained from Asp718- or the HindIII-digestion were labelled to an extent which was proportional to their length. Two of the HindIII fragments which were located near the ends of the genome appeared to have more intense labelling. By contrast,

Asp718 fragments from similar positions in the genome did not appear to show preferential labelling of this kind. All in all, the 8-methoxypsoralen crosslinkings were uniformly distributed over the virus genome.

Example 14

Determining the radiation dose required for inactivation of tumour cells

6 average tissue culture flasks (T75) each containing 6 x 10^5 human melanoma cells in 20 ml of normal culture medium were irradiated after one day with 5, 10, 20, 25, 50, 75 or 100 Gy; 6 flasks were not irradiated. A radiation apparatus known as Gammacell 40, No. 126, Type B(U), (made by Nordion International INC) was used, having 2 gamma cells of Cs-137 which together yield about 72.5 Gy/h. 6 days after irradiation the cells from one flask in each group were trypsinised and centrifuged, the pellet was taken up, 100 μl of cell suspension were mixed with trypan blue and counted in the counter chamber. The remaining cells were mixed with new medium and poured into a new culture flask (1st passage). One week later the process was repeated; one flask from each group was trypsinised and counted and the 1st passage was also counted as a control. The non-irradiated cells and those irradiated at 5 Gy showed significant growth. After further intervals of one week the process was repeated and the test was concluded after a total of 6 weeks. For the growth curves the highest number of cells counted was used (in the original or control flasks). From a radiation dosage of 20 Gy, neither the original flasks nor the control flasks shows any cell growth. At 10 Gy growth was observed in the original flasks. The growth curves are shown in Fig. 24. On the basis of the values obtained

from three different melanoma cell cultures, a dosage of 100 Gy was selected as a safe radiation dosage for all experiments.

Example 15

Detection of plasmid- and adenovirus-DNA *in vivo* using polymerase chain reaction (PCR)

The following methods were used in this Example:

Injection of the cancer vaccines:
M3 melanoma cells were transfected with recombinant mouse-interleukin-2 plasmid using the adenovirus transferrin method as described in Example 8 c). After 24 hours the cytokine production was determined (20,000-50,000 units of IL-2 in 24 hours per million cells). The cells were trypsinised, washed in serum free-medium and adjusted to a concentration of 3×10^5 or 1×10^6 cells per 100 µl of isotonic saline solution. 100 µl of cell suspension were injected by means of a cannula into the right flank of DBA/2 mice by subcutaneous route (see also Examples 7 and 8).

Purification of the tissue samples:
At various times after the injection the mice were killed and the injection site and various tissue samples were removed. These samples were mechanically ground up and incubated overnight in 1 ml of proteinase K buffer (50 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 1 % SDS, 0.5 mg/ml proteinase K) at 55°C. Then the DNA was extracted with phenol/chloroform and precipitated with 0.5 volumes of isopropanol. The samples which contained the total cellular DNA were washed with 70% ethanol and taken up in TE buffer.

In order to purify peripheral mononuclear blood cells (PMBCs) heart punctation was carried out. The blood mixed with citrate buffer (10 mM) was then separated using a Ficoll-gradient and the PMBCs were digested with proteinase K buffer. DNA purification was carried out as described above.

Polymerase chain reaction:
The reaction mixture was made up of 1 µg of DNA, 1 x PCR buffer (Boehringer Mannheim), 1 mM final concentration of deoxy-nucleotide, 3 units of Taq-polymerase (Boehringer Mannheim) and 25 pmol of the specific primer. In the case of the interleukin-2 plasmid amplification, 1,000 copies of the internal control were additionally added. PCR reaction times were: 5 minutes at 95°C for denaturing, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C, 2 mins at 72°C. Then the amplification products were mixed with sample buffer and separated in a 2% agarose gel.
The specific primers had the following sequences:
IL-2 up (Position 99-122) (SEQ ID NO:3)
GTCAACAGCGCACCCACTTCAAGC.
IL-2 down (Position 548-526) (SEQ ID NO:4)
GCTTGTTGAGATGATGCTTTGACA.
Adeno up (Position 1228-1248) (SEQ ID NO:5)
GGTCCTGTGTCTGAACCTGAG.
Adeno down (Position 1545-1525) (SEQ ID NO:6)
TTATGGCCTGGGGCGTTTACA.
(In the sequence protocol, for reasons of the programme, referring to the synthetic oligonucleotides the abbreviation "cDNS" is used throughout for the type of molecule and "5'UTR" as the key.)
The detection limits of the amplification products are between 200 and 1,000 copies of DNA depending on the test.

a) In order to detect recombinant IL-2 DNA and

adenovirus dl1014 DNA at the injection site at different times after vaccination, nine DBA/2 mice were each immunised with 3 x 10^5 IL-2 plasmid-transfected M3 melanoma cells. After 1, 2 and 5 days, 3 mice were killed and the immunisation sites were excised. PCR-analysis showed that after only 2 days considerable breakdown of the IL-2 DNA has set in and after 5 days this DNA can no longer be detected. In an analogous experiment, IL-2 plasmid DNA could be detected from the injection site of an animal on day 5 after the injection. In PCR-reactions with the adenovirus-specific primers, adenovirus-DNA could be detected in samples taken on day 1 and day 2 but not in samples taken on day 5.

b) In order to detect IL-2 DNA and adenovirus dl1014 DNA from peripheral mononuclear blood cells 24 and 48 h after vaccination, twelve DBA/2 mice were each immunised with 1 x 10^6 IL-2-plasmid-transfected M3 melanoma cells. After 24 and 48 hours, a period in which there is massive breakdown of the M3 cells at the injection site, 6 mice were killed and blood samples were taken. PCR-analysis showed that, at both times, there was neither specific IL-2 DNA nor adenovirus-DNA detectable in the blood.

c) In order to detect IL-2 DNA and adenovirus dl1014 DNA from tissue samples from various organs, six DBA/2 mice were each immunised with 1 x 10^6 IL-2-plasmid-transfected M3 melanoma cells. After 24 and 48 hours 3 animals were killed and tissue samples were taken from the injection site, the lymph node leading off it, the spleen, kidneys, liver, colon and ovaries. Specific amplification products could only be detected at the injection sites. All the tissue samples tested were negative.

d) In order to rule out the possibility of transfer of recombinant IL-2 DNA into the germ cells, six female and six male DBA/2 mice were immunised with 1 x 10^6 IL-2-plasmid-transfected M3 melanoma cells. After 24 and 48 hours 3 female and 3 male animals were killed and the germ cells were isolated. PCR-analysis showed that no transfer into the germ cells had taken place.

Example 16

a) Testing of the efficacy of the cancer vaccines for their protective effect against metastasis formation ("therapeutic mouse model")

In this Example the same procedures were used as in Example 8 with regard to the transfection complexes used, the culturing of the cells and the transfection methods. All the test animals were mice of the C57BL/6J strain, 8 animals being used per group. The melanoma cells used were the cells B16-F10 which are syngenic for the strain of mouse used (NIH DCT Tumor Depository; Fidler et al., 1975).

On day 1, 1 x 10^5 living B16-F10 cells were injected intravenously into the experimental animals in order to produce the formation of metastases. On days 4, 11 and 17 the cancer vaccines were administered subcutaneously in order to achieve immunisation against the metastases.

For each immunisation, 1 x 10^5 irradiated cells were injected, the vaccines containing different amounts of cells which had been transfected with cytokine-plasmid ("transfected cells" hereinafter refers to cells which have been subjected to a transfection treatment; the expression values for the cytokines are given per mouse in 24 hours; the terms used are as in Fig. 25):

1) Controls:

a) Cells which are only irradiated ("irradiated")

b) Irradiated cells transfected with the empty vector pSP ("empty vector")

2) Cells transfected with the IL-2 plasmid:

a) 100% transfected cells (expression: 15,000 units; "IL-2 high")

b) 20% transfected cells, 80% non-transfected cells (expression: 3,000 - 4,000 units; "IL-2 medium")

c) 2% transfected cells, 98% non-transfected cells (expression: 400 units; "IL-2 low")

3) Cells transfected with the GM-CSF-plasmid:

a) 100% transfected cells (expression: 500 ng; "GM-CSF high")

b) 10% transfected cells, 90% non-transfected cells (expression: 50 ng; "GM-CSF medium")

c) 1% transfected cells, 99% non-transfected cells (expression: 5 ng; "GM-CSF low")

4) Cells transfected with the IFN-γ-plasmid:

a) 100% transfected cells (expression: 1,000 ng; "IFN-γ high")

b) 10% transfected cells, 90% non-transfected cells (expression: 100 ng; "IFN-γ medium")

c) 1% transfected cells, 99% non-transfected cells (expression: 10 ng; "IFN-γ low")

On day 28 the protective effect of the cancer vaccines against metastasis formation was analysed by visually inspecting the mice for the presence of tumours.

The test results shown in Fig. 25 show that the cancer vaccines act as a function of the transfected gene and depending on the expression level of the gene.

b) Testing of the cancer vaccines for their ability to eliminate artificially induced "micrometastases"

In order to find the tumorigenic dose of M3-cells, first of all a preliminary test was carried out to determine the number of cells which lead to local tumour development in 50% of all animals with 8 weeks and in 100% of all animals within 10 weeks, using 1×10^3 or 3×10^3 M3-melanoma cells.

On day 0 all the test animals were given a subcutaneous injection of 5×10^3 viable M3-cells; the M3-cells which do not metastasise can be regarded as "micrometastasis". The administration of the tumour vaccines was carried out in all the experimental animals after 1, 2 and 5 weeks. The cytokine expression of the tumour vaccines administered was, per mouse, for IL-2, 1,020 units for the 1st immunisation, 1,870 units for the 2nd immunisation and 1,400 units for the 3rd immunisation. For GM-CSF the values per vaccine and mouse were 14 ng, 9 ng and 22 ng. The test groups were each made up of 10 animals, the animals in the first group having been immunised with 1×10^5 M3-cells which were transfected with the vector pWS2m and irradiated. The animals in the second group were immunised with 1×10^5 M3-cells which were transfected with the vector pWE-Gm and irradiated. The first control group was treated with 1×10^5 M3-cells which were irradiated but not transfected. The second control group was given 5×10^3 M3-cells as a control for the tumour development. The animals were observed over a period of more than 4 months. It was found that in the two groups which had been given cells expressing a cytokine as the tumour vaccine, 80% of the animals were protected from tumour development. In the first control group all the animals developed tumours within 8 weeks. In the second control group all the animals except one developed tumours.

Example 17

Effectiveness of the tumour vaccines as a function of the cytokine dosage in the prophylactic mouse model

In this Example, the same procedures were used as in Example 8, with regard to the transfection complexes used, the culturing of the cells and the transfection methods. DBA/2 mice and M3 melanoma cells were used. The cytokine plasmids used were the same as in Example 16. The immunisation was carried out as described in Example 8 and for setting the tumour ("Challenge") 3 x 10^5 cells were used instead of 1 x 10^5 cells as in Example 8. The mixing ratios of transfected cells and cells which were merely irradiated and the expression values corresponded to those in Example 16. After the challenge the animals were investigated for 8 weeks as to the presence of tumours; the results of these tests are given in Fig. 26.

Example 18

Use of endosomolytic peptides for preparing cancer vaccines

a) Synthesis of peptide INF5

The peptide designated INF5 (SEQ ID NO:7) was synthesised by the HBTU activation method (Knorr et al., 1989; 1 mmol scale), using 230 mg of TentaGel S-PHB resin (Rapp polymer; 0.27 mmol/g) as the solid phase. The first amino acid which was coupled was N-α-N-ε-di-Fmoc-lysine. This yielded a head-to-head dimer with a C-terminal lysine as the connecting amino acid. (The following side chain protecting groups were used: (Trt)Asn, (Trt)Cys or (t-Bu)Cys, (t-Bu)Glu, (Trt)His,

(t-Bu)Ser. The peptides were cleaved by the resin and the side protecting groups apart from (t-Bu)Cys were removed by treating 10 - 20 mg of peptide-charged resin with 1 ml of a mixture of trifluoroacetic acid/water/phenol/thioanisole/ethanedithiol (10:0.5:0.75:0.5:0.25) for 1.5 hours at ambient temperature.) For precipitating the peptide the cleaved mixture was pipetted dropwise into 40 ml of ether with stirring and the mixture was left to stand for 1 hour. The crude peptide was obtained by centrifuging, then washed with ether and dried under Argon and finally under a high vacuum.

b) Gene transfer into human melanoma cells using INF5

i) Expression of the luciferase-reporter gene

First of all, preliminary tests were carried out with the reporter gene construct pCMVL, using 1.5 µg of TfpL290, 5 µg of pL290, 40 µg of INF5 and 3 µg of pCMVL to prepare transfection complexes. In these complexes the peptide was ionically bound to polylysine. The DNA-complexes were mixed with 0.5 ml of RPMI 1640 (Gibco), containing 10 % FCS, and applied to M3-melanoma cells (1×10^5 cells in 6-well-plates). After 4 hours the medium was replaced by fresh medium. 24 hours after transfection the cells were harvested and investigated for luciferase activity. Expression was detected corresponding to 12,866,000 light units.

ii) Expression of human IL-2

Transfection complexes consisting of 3 µg of pGShIL-2tet, 1.5 µg of TfpL290, 5 µg of pL290 and 40 µg of INF5 were applied to melanoma cells as described in i). One and two days after transfection the quantities of IL-2 secreted into the culture medium within 24 hours were

measured by ELISA assay (Biokine IL-2 Test kit, T Cell Diagnostics). The values were 6,500 BRMP units on the first day and 11,500 BRMP units on the second day, one unit corresponding to 40 pg of IL-2.

c) Testing of tumour cells transfected by means of INF5 as tumour vaccines in the prophylactic melanoma mouse model

In this experiment, mice of the C57BL/6J strain and B16-F10-cells were used. Two immunisations were carried out at an interval of 7 days using 1 x 10^5 cells; 7 days after the last immunisation the challenge was set (1 x 10^5 cells). The cancer vaccines were prepared using a transfection complex consisting of 6 μg of pWS-Gm-DNA, 3 μg of TfpL, 10 μg of pL and 40 μg of INF5. Tumour vaccines were used containing different amounts of cells carrying GM-CSF plasmid, the mixing ratios of irradiated, transfected cells and cells which had only been irradiated being the same as in Example 16. The results with tumour vaccines which contained cells transfected by means of INF-5 are given in Fig. 27. There was found to be a protective effect against tumour formation at the high and medium GM-CSF dose.

Example 19

Interleukin expression in human melanoma cells

One week after surgical removal, 3 x 10^5 melanoma cells in 6 cm cell culture dishes were transfected with 2 ml of transfection complex (0.5 ml of starting solution, containing biotinylated 8-methoxypsoralen/UV-inactivated adenovirus (0.54 x 10^{12} particles/ml), 100 ng/μl of streptavidin-polylysine, 6 μg of plasmid-DNA pGShIL-2tet and 1 μg/μl of TfpL, in HBS, diluted with 1.5 ml of

medium). The experiments were carried out with irradiated (100 Gy) and non-irradiated cells. The IL-2 values were measured after the times specified in Fig. 28; the IL-2 values related to 10^6 cells and 24 hours.

Example 20

Development of a galenic formulation for the cancer vaccines

The starting materials were the MM3-melanoma cells transfected with the plasmid pGShIL-2tet and irradiated with 100 Gy; after 20 hours' incubation at 37°C the cells were trypsinised and the cell number and viability were determined using trypan blue. Then the cells were divided up into four equal groups in order to test four different freezing media. The four sample groups were centrifuged at 800 rpm (120 g) in RPMI 1640 before being mixed with freezing medium and the cell pellets were placed in 1 ml of freezing medium. After aliquots had been removed for determining the cell number and viability, the samples were immediately placed in the freezing apparatus and carefully frozen to -100°C using a temperature gradient programme (-1°C/min) and transferred into liquid nitrogen. The freezing media tested were: Medium 1: 70 % RPMI, 20 % FCS, 10 % DMSO; Medium 2: 20 % human serum albumin, 10 % DMSO; Medium 3: 12 % HES (hydroxyethylstarch), Ringer solution (Leopold Pharma, No. 2870), 5 % DMSO; Medium 4: 12 % HES in Ringer solution.

After 38 days the cells were thawed and immediately afterwards the cell number and viability of the cells were determined using trypan blue. Then the samples were washed with the corresponding medium (freezing medium without DMSO) in a first washing step and with

Ringer solution in a second and third washing step, then centrifuged and resuspended in Ringer solution. Then the cell number and viability of the cells were determined once more. In addition, the cell number and viability were determined after 24 and after 48 hours at 4°C. The results of these tests are shown in Table V.

From each sample group (Medium 1 - 4), additionally, 300 µl with 3 ml of complete medium were placed in T25-culture flasks and incubated at 37°C; after 24 and 48 hours the supernatant was removed in order to determine the IL-2 expression (sample group Medium 1) and to determined the number of cells in the supernatant. Of the other samples, which had initially been left to stand at 4°C in Ringer solution, an aliquot of 300 µl was also taken after 24 and 48 hours and incubated at 37°C as described above (IL-2 determination for sample group Medium 1). The results of these tests ("4° galenic") are given in Table VI.

Example 21

Influence of the endotoxin content of the DNA on the expression of IL-2 in primary human melanoma cells

In this Example the following materials and methods were used:

a) DNA preparation

The plasmid pGShIL-2tet was obtained from overnight cultures of *E. coli* (cultured in the presence of 5 µg/ml tetracyclin in LB medium).

b) Purification of plasmid-DNA of endotoxin (lipopolysaccharide)

i) Triton X-114 extraction

In order to obtain a homogeneous preparation of the detergent, Triton X-114 (Sigma) was subjected to three 0°C/30°C temperature cycles as described by Bordier, 1981. The extraction of the lipopolysaccharides from the DNA sample was carried out as follows, as a modification of published methods (Aida and Pabst, 1990; Manthorpe et al., 1993): the DNA sample (0.5 - 1.5 mg/ml in 10 mM Tris, 0.1 mM EDTA, pH 7.4 (TE)) was applied to 0.3 M sodium acetate (pH 7.5). Then 3 µl of Triton X-114 were added per 100 µl of DNA solution, the samples were mixed thoroughly in a vortex and incubated on ice for 10 minutes. To enable the two phases to separate, the samples were stored for 5 minutes at 30°C, centrifuged in a pre-heated Eppendorf centrifuge at 2,000 rpm for 2 minutes and the aqueous phase was placed in a fresh Eppendorf test tube. This extraction was carried out twice more and the aqueous phase finally obtained was precipitated at ambient temperature with 0.6 volumes isopropanol, the precipitate was recovered by centrifuging, washed twice with 80% ethanol, dried in air, taken up in TE once more and the quantity was determined. In order to do this, the sample was treated with RNase A, proteinase K, phenol/chloroform and chloroform, precipitated again and the final DNA pellet was suspended in TE and the absorption was determined at 260 nm, starting with the assumption that a concentration of 0.05 mg/ml of DNA has an absorption value of 1.

ii) Polymyxin chromatography

One volume of polymyxin resin sludge (Affi-Prep-Polymyxin, Biorad) corresponding to the volume of the DNA sample was briefly mixed with three volumes of 0.1 N NaOH, then washed three times with five resin volumes

TE. The pelleted resin was taken up again with the DNA samples (in TE 0.8 - 1.2 mg/ml) and the mixture was stirred overnight at 4°C. Then the sample was placed on a disposable column pretreated with 0.1 NaOH and washed with TE. The eluate was collected, the resin was washed with another volume of TE and the eluate was combined with the washing liquid. The DNA of this pooled sample was precipitated with 1/10 volume 3 M sodium acetate, pH 5, and 2 volumes of ethanol. The further treatment of the precipitate and the DNA measurement were carried out as described above.

c) Cell culture

Primary human melanoma cells were isolated and cultivated in RPMI 1640 medium (Gibco/BRL), supplemented with 100 I.U./ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, 1% sodium pyruvate and 10% heat-inactivated FCS.

d) Endotoxin assay

The lipopolysaccharide content was determined using the chromogenic limulus assay which is based on the limulus coagulation reaction of amoebocytes (Iwanaga, 1993; obtainable from BioWhittaker QCL-1000). Before the test was carried out all the biological materials and reagents used were tested to ensure that they were free from lipopolysaccharide (<0.1 endotoxin units (EU)/50 µl of solution).

e) Preparation of transfection complexes

Ternary complexes of transferrin-polylysine, streptavidin-polylysine and biotinylated, 8-methoxypsoralen/UV-inactivated adenovirus (dl1014 (8 µl, 1 x 10^{12} particles/ml) and plasmid DNA (6 µg, diluted in

100 µl with the lipopolysaccharide content specified) were prepared as described in the preceding Examples.

Primary human melanoma cells designated H225 (2 x 10^5 cells/6 cm culture dish) were transfected with 6 µg of the plasmid purified by various methods in accordance with the data in Fig. 29. The endotoxin content of the plasmid preparation before purification is shown as a dark shaded bar in the Figure. After purification using polymyxin resin or extraction with Triton X-114 all the preparations contained less than 0.1 EU of lipopolysaccharide/6 µg of DNA. The IL-2 content in the cell supernatant was measured using ELISA (T Cell Diagnostics Inc., Cambridge, MA USA), the values shown in the Figures indicating units/10^6 cells over 24 hours.

Within the scope of this Example, tests were also carried out which showed that the fall in expression caused by the addition of LPS to purified DNA can be at least partially cancelled out if polymyxin is added to the medium.

Example 22

Induction of a systemic immune response by immunisation with cytokine-transfected, irradiated colon carcinoma cells ("prophylactic colon carcinoma model")

In this Example, the same methods are used as in Example 8 with regard to the transfection complexes used, the cultivation of the cells and the transfection methods, whilst the details in Example 16 were followed with regard to the cytokine DNA used and the dosage thereof as well as the controls (empty vector, cells irradiated only); the terms used in Fig. 30 correspond to those of Example 16 and Fig. 25. Cells of a colon carcinoma cell

line designated CT 26 were used, the establishment of which was described by Brattain et al., 1988. The experimental animals were mice of the BALB/c strain. 1 x 10^5 cells were used for the two immunisations and the challenge was set with 3 x 10^5 cells. In Table VII the expression values are given over 24 hours for IL-2 (units/mouse), IFN-gamma and GM-CSF (in each case ng/mouse), secreted between the irradiation of the cells and injection (1st immunisation/2nd immunisation). Fig. 30 shows a protective effective of the colon carcinoma tumour vaccines against tumour formation.

Table I

Plasmid ratio	Gene expression	
	ng IFN-γ (per 10^6 cells/24 h)	units IL-2
IFN-γ 100 %/IL-2 0 %	369.6	-
IFN-γ 75 %/IL-2 25 %	290.4	7920
IFN-γ 50 %/IL-2 50 %	204.6	11880
IFN-γ 25 %/IL-2 75 %	83.8	14520
IFN-γ 10 %/IL-2 90 %	42.9	18150
	Luciferase light units/μg of protein	
pCMVL 100 %/pSP 0 %	1531438	
pCMVL 75 %/pSP 25 %	1191660	
pCMVL 50 %/pSP 50 %	700052	
pCMVL 25 %/pSP 75 %	209914	
pCMVL 12.5 %/pSP 87.5 %	48352	
pCMVL 6.3 %/pSP 93.7 %	14632	
pCMVL 3.1 %/pSP 96.9 %	7324	

Table II

M-3 Melanoma development in DBA/2 mice

Immunisations (2x)	Weeks after tumour cell implantation									
(1 x 10^5 cells, irradiated)	1w	2w	3w	4w	5w	6w	7w	8w	9w	10w
No immunisation	2/4	4/4	4/4	4/4	4/4	4/4				
Not transfected M-3	0/6	2/6	4/6	4/6	3/6	3/6	4/6	4/6	4/6	5/6
pSP 100% (dl1014)	0/6	3/6	3/6	3/6	3/6	3/6	3/6	4/6	4/6	4/6
IL-2 100% (dl1014)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Table III

M-3 Melanoma development in DBA/2 mice

Immunisations (2x)	Weeks after tumour cell implantation								
(1 x 10^5 cells, irradiated)	1w	2w	3w	4w	5w	6w	7w	8w	9w
No immunisation	6/6	6/6	6/6	6/6	6/6	6/6	6/6		
IL-2 100% (dl1014)	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
IL-2 10% (dl1014)	0/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5	2/5
IL-2 90%+IFN-γ 10% (dl1014)	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
IFN-γ 10% (dl1014)	4/6	5/6	5/6	5/6	5/6	5/6	5/6	5/6	5/6
IL-2 100% (dl312)	1/5	1/5	1/5	1/5	1/5	1/5	1/5	2/5	2/5

Table IV

Tumour development in DBA/2 mice

Immunisations (2 x) (1×10^5 M-3 cells)	Weeks after tumour cell implantation 1 w	2 w	3 w	4 w
		1×10^5 M-3		
No immunisation	0/6	2/6	6/6	6/6
pSP (dl1014)	0/6	1/6	3/6	4/6
IL-2 100 % (dl1014)	0/6	0/6	0/6	0/6
IL-2 4 % (dl1014)	0/5	0/5	1/5	1/5
		3×10^5 M-3		
IL-2 100 % (dl1014)	0/5	0/5	0/5	2/5
IL-2 4 % (dl1014)	0/5	2/5	3/5	4/5
		1×10^6 M-3		
IL-2 100 % (dl1014)	0/4	0/4	1/4	1/3
IL-2 4 % (dl1014)	1/5	3/5	4/5	4/5
		1×10^5 KLN 205		
IL-2 100 % (dl1014)	0/6	2/6	6/6	6/6

Table V

FROZEN GALENIC PREPARATIONS

Cells: MM3 transfected with human IL-2 irradiated with 100 grey, N_2-frozen, thawed 38 days later

Time	Immediately before freezing		Immediately after thawing		Galenic preparation (Ringers) 0 h		24 h at 4°C		48 h at 4°C	
Freezing medium	No. of cells ($x10^6$)	Viability (%)	No. of cells ($x10^6$)	Viability (%)	No. of cells ($x10^6$)	Viability (%)	No. of cells ($x10^6$)	Viability (%)	No. of cells ($x10^6$)	Viability (%)
70% RPMI + 20% FCS + 10% DMSO	3.1	99%	2.8	98%	1.6	80%	1.7	77%	1.7	80%
20% HSA + 10% DMSO	3.0	100%	2.9	98%	0.8	62%	0.5	31	0.4	30%
12% HES, Ringers + 5%DMSO	2.9	98%	2.0	86%	0.8	50%	0.82	50	0.8	53%
12% HES in Ringers	2.8	94%	0.4	40%	0.3	47%	nd	nd	nd	nd

Table VI

MM3 cells, 4°C galenic preparation seeded out at time:	0 h at 4°C		24 h at 4°C		48 h at 4°C	
Freezing medium	Appearance (% cells in supernatant)	IL-2 expression Units/24 h/ $1x10^6$ cells	Appearance (% cells in supernatant	IL-2 expression Units/24 h/ $1x10^6$ cells	Appearance (% cells in supernatant	IL-2 expression Units/24 h/ $1x10^6$ cells
70% RPMI + 20% FCS + 10% DMSO	24 h: 10% 48 h: 10%	24 h: 11 195 48 h: 7069	24 h: 50% 48 h: 50%	24 h: 4646 48 h: 4862	24 h: 50% 48 h: 60%	24 h: 5940 48 h: 3794
20% HSA + 10% DMSO	24 h: 70% 48 h: 80%		24 h: 95% 48 h: 95%		24h: 100% 48 h: 100%	
12% HES in Ringers + 5% DMSO	24 h: 20% 48 h: 20%		24 h: 60% 48 h: 60%		24 h: 70% 48 h: 70%	
12% HES in Ringers	24 h: 40% 48 h: 40%					

Table VII

Vaccine	Expression 1st Immunisation/2nd Immunisation
GM-CSF (high)	241/184
GM-CSF (medium)	24/18
GM-CSF (low)	2.4/1.8
IL-2 (high)	4302/4624
IL-2 (medium)	860/925
IL-2 (low)	86/93
IFN-γ (high)	129/63
IFN-γ (medium)	13/6
IFN-γ (low)	1.3/0.6

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Boehringer Ingelheim International GmbH

(B) STREET: Binger Strasse 173

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(G) TELEPHONE: 06132/772822

(H) TELEFAX: 06132/774377

(I) TELEX: 4187910 bi d

(ii) TITLE OF APPLICATION: Process for preparing cancer vaccines

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER-READABLE FORM:

(A) DATA CARRIER: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPA)

(2) INFORMATION ON SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1664 Base pairs

(B) TYEP: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: cDNA TO mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTISENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rabies virus

(ix) FEATURES:
(A) NAME/KEY: 5'UTR
(B) POSITION: 1..6

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) POSITION: 7..1581

(ix) FEATURES:
(A) NAME/KEY: sig_peptide
(B) POSITION: 7..63

(ix) FEATURES:
(A) NAME/KEY: mat_peptide
(B) POSITION: 64..1578

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TCTAAT ATG GTT CCT CAG GCT CTC CTG TTT GTA CCC CTT CTG GTT TTT    48
       Met Val Pro Gln Ala Leu Leu Phe Val Pro Leu Leu Val Phe
       -19             -15                 -10

CCA TTG TGT TTT GGG AAA TTC CCT ATT TAC ACG ATC CCA GAC AAG CTT   96
Pro Leu Cys Phe Gly Lys Phe Pro Ile Tyr Thr Ile Pro Asp Lys Leu
 -5                  1               5                  10

GGT CCC TGG AGC CCG ATT GAC ATA CAT CAC CTC AGC TGC CCA AAC AAT  144
Gly Pro Trp Ser Pro Ile Asp Ile His His Leu Ser Cys Pro Asn Asn
            15                  20                  25

TTG GTA GTG GAG GAC GAA GGA TGC ACC AAC CTG TCA GGG TTC TCC TAC  192
Leu Val Val Glu Asp Glu Gly Cys Thr Asn Leu Ser Gly Phe Ser Tyr
        30                  35                  40
```

```
ATG GAA CTT AAA GTT GGA TAC ATC TTA GCC ATA AAA ATG AAC GGG TTC  240
Met Glu Leu Lys Val Gly Tyr Ile Leu Ala Ile Lys Met Asn Gly Phe
     45                  50                  55

ACT TGC ACA GGC GTT GTG ACG GAG GCT GAA ACC TAC ACT AAC TTC GTT  288
Thr Cys Thr Gly Val Val Thr Glu Ala Glu Thr Tyr Thr Asn Phe Val
 60                  65                  70                  75

GGT TAT GTC ACA ACC ACG TTC AAA AGA AAG CAT TTC CGC CCA ACA CCA  336
Gly Tyr Val Thr Thr Thr Phe Lys Arg Lys His Phe Arg Pro Thr Pro
                 80                  85                  90

GAT GCA TGT AGA GCC GCG TAC AAC TGG AAG ATG GCC GGT GAC CCC AGA  384
Asp Ala Cys Arg Ala Ala Tyr Asn Trp Lys Met Ala Gly Asp Pro Arg
            95                 100                 105

TAT GAA GAG TCT CTA CAC AAT CCG TAC CCT GAC TAC CGC TGG CTT CGA  432
Tyr Glu Glu Ser Leu His Asn Pro Tyr Pro Asp Tyr Arg Trp Leu Arg
        110                 115                 120

ACT GTA AAA ACC ACC AAG GAG TCT CTC GTT ATC ATA TCT CCA AGT GTA  480
Thr Val Lys Thr Thr Lys Glu Ser Leu Val Ile Ile Ser Pro Ser Val
    125                 130                 135

GCA GAT TTG GAC CCA TAT GAC AGA TCC CTT CAC TCG AGG GTC TTC CCT  528
Ala Asp Leu Asp Pro Tyr Asp Arg Ser Leu His Ser Arg Val Phe Pro
140                 145                 150                 155

AGC GGG AAG TGC TCA GGA GTA GCG GTG TCT TCT ACC TAC TGC TCC ACT  576
Ser Gly Lys Cys Ser Gly Val Ala Val Ser Ser Thr Tyr Cys Ser Thr
                160                 165                 170

AAC CAC GAT TAC ACC ATT TGG ATG CCC GAG AAT CCG AGA CTA GGG ATG  624
Asn His Asp Tyr Thr Ile Trp Met Pro Glu Asn Pro Arg Leu Gly Met
            175                 180                 185

TCT TGT GAC ATT TTT ACC AAT AGT AGA GGG AAG AGA GCA TCC AAA GGG  672
Ser Cys Asp Ile Phe Thr Asn Ser Arg Gly Lys Arg Ala Ser Lys Gly
        190                 195                 200

AGT GAG ACT TGC GGC TTT GTA GAT GAA AGA GGC CTA TAT AAG TCT TTA  720
Ser Glu Thr Cys Gly Phe Val Asp Glu Arg Gly Leu Tyr Lys Ser Leu
    205                 210                 215

AAA GGA GCA TGC AAA CTC AAG TTA TGT GGA GTT CTA GGA CTT AGA CTT  768
Lys Gly Ala Cys Lys Leu Lys Leu Cys Gly Val Leu Gly Leu Arg Leu
220                 225                 230                 235

ATG GAT GGA ACA TGG GTC GCG ATG CAA ACA TCA AAT GAA ACC AAA TGG  816
Met Asp Gly Thr Trp Val Ala Met Gln Thr Ser Asn Glu Thr Lys Trp
                240                 245                 250

TGC CCT CCC GAT CAG TTG GTG AAC CTG CAC GAC TTT CGC TCA GAC GAA  864
Cys Pro Pro Asp Gln Leu Val Asn Leu His Asp Phe Arg Ser Asp Glu
```

```
                255                 260                 265

ATT GAG CAC CTT GTT GTA GAG GAG TTG GTC AGG AAG AGA GAG GAG TGT  912
Ile Glu His Leu Val Val Glu Glu Leu Val Arg Lys Arg Glu Glu Cys
        270                 275                 280

CTG GAT GCA CTA GAG TCC ATC ATG ACA ACC AAG TCA GTG AGT TTC AGA  960
Leu Asp Ala Leu Glu Ser Ile Met Thr Thr Lys Ser Val Ser Phe Arg
    285                 290                 295

CGT CTC AGT CAT TTA AGA AAA CTT GTC CCT GGG TTT GGA AAA GCA TAT 1008
Arg Leu Ser His Leu Arg Lys Leu Val Pro Gly Phe Gly Lys Ala Tyr
300                 305                 310                 315

ACC ATA TTC AAC AAG ACC TTG ATG GAA GCC GAT GCT CAC TAC AAG TCA 1056
Thr Ile Phe Asn Lys Thr Leu Met Glu Ala Asp Ala His Tyr Lys Ser
                320                 325                 330

GTC AGA ACT TGG AAT GAG ATC CTC CCT TCA AAA GGG TGT TTA AGA GTT 1104
Val Arg Thr Trp Asn Glu Ile Leu Pro Ser Lys Gly Cys Leu Arg Val
            335                 340                 345

GGG GGG AGG TGT CAT CCT CAT GTG AAC GGG GTG TTT TTC AAT GGT ATA 1152
Gly Gly Arg Cys His Pro His Val Asn Gly Val Phe Phe Asn Gly Ile
        350                 355                 360

ATA TTA GGA CCT GAC GGC AAT GTC TTA ATC CCA GAG ATG CAA TCA TCC 1200
Ile Leu Gly Pro Asp Gly Asn Val Leu Ile Pro Glu Met Gln Ser Ser
    365                 370                 375

CTC CTC CAG CAA CAT ATG GAG TTG TTG GAA TCC TCG GTT ATC CCC CTT 1248
Leu Leu Gln Gln His Met Glu Leu Leu Glu Ser Ser Val Ile Pro Leu
380                 385                 390                 395

GTG CAC CCC CTG GCA GAC CCG TCT ACC GTT TTC AAG GAC GGT GAC GAG 1296
Val His Pro Leu Ala Asp Pro Ser Thr Val Phe Lys Asp Gly Asp Glu
                400                 405                 410

GCT GAG GAT TTT GTT GAA GTT CAC CTT CCC GAT GTG CAC AAT CAG GTC 1344
Ala Glu Asp Phe Val Glu Val His Leu Pro Asp Val His Asn Gln Val
            415                 420                 425

TCA GGA GTT GAC TTG GGT CTC CCG AAC TGG GGG AAG TAT GTA TTA CTG 1392
Ser Gly Val Asp Leu Gly Leu Pro Asn Trp Gly Lys Tyr Val Leu Leu
        430                 435                 440

AGT GCA GGG GCC CTG ACT GCC TTG ATG TTG ATA ATT TTC CTG ATG ACA 1440
Ser Ala Gly Ala Leu Thr Ala Leu Met Leu Ile Ile Phe Leu Met Thr
    445                 450                 455

TGT TGT AGA AGA GTC AAT CGA TCA GAA CCT ACG CAA CAC AAT CTC AGA 1488
Cys Cys Arg Arg Val Asn Arg Ser Glu Pro Thr Gln His Asn Leu Arg
460                 465                 470                 475
```

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GGG ACA GGG AGG GAG GTG TCA GTC ACT CCC CAA AGC GGG AAG ATC ATA   1536
Gly Thr Gly Arg Glu Val Ser Val Thr Pro Gln Ser Gly Lys Ile Ile
                480                 485                 490

TCT TCA TGG GAA TCA CAC AAG AGT GGG GGT GAG ACC AGA CTG TGAGGACTGG 1588
Ser Ser Trp Glu Ser His Lys Ser Gly Gly Glu Thr Arg Leu
            495                 500                 505

CCGTCCTTTC AACGATCCAA GTCCTGAAGA TCACCTCCCC TTGGGGGGTT CTTTTTGAAA 1648

AAAAAAAAAA AAAAAA                                                1664
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(2) INFORMATION ON SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 524 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Protein

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO: 2:

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Met Val Pro Gln Ala Leu Leu Phe Val Pro Leu Leu Val Phe Pro Leu
-19             -15                 -10                 -5

Cys Phe Gly Lys Phe Pro Ile Tyr Thr Ile Pro Asp Lys Leu Gly Pro
             1               5                  10

Trp Ser Pro Ile Asp Ile His His Leu Ser Cys Pro Asn Asn Leu Val
     15                  20                  25

Val Glu Asp Glu Gly Cys Thr Asn Leu Ser Gly Phe Ser Tyr Met Glu
 30                  35                  40                  45

Leu Lys Val Gly Tyr Ile Leu Ala Ile Lys Met Asn Gly Phe Thr Cys
                 50                  55                  60

Thr Gly Val Val Thr Glu Ala Glu Thr Tyr Thr Asn Phe Val Gly Tyr
             65                  70                  75

Val Thr Thr Thr Phe Lys Arg Lys His Phe Arg Pro Thr Pro Asp Ala
         80                  85                  90

Cys Arg Ala Ala Tyr Asn Trp Lys Met Ala Gly Asp Pro Arg Tyr Glu
     95                  100                 105

Glu Ser Leu His Asn Pro Tyr Pro Asp Tyr Arg Trp Leu Arg Thr Val
110                 115                 120                 125

Lys Thr Thr Lys Glu Ser Leu Val Ile Ile Ser Pro Ser Val Ala Asp
                130                 135                 140
```

Leu Asp Pro Tyr Asp Arg Ser Leu His Ser Arg Val Phe Pro Ser Gly
145 150 155

Lys Cys Ser Gly Val Ala Val Ser Ser Thr Tyr Cys Ser Thr Asn His
160 165 170

Asp Tyr Thr Ile Trp Met Pro Glu Asn Pro Arg Leu Gly Met Ser Cys
175 180 185

Asp Ile Phe Thr Asn Ser Arg Gly Lys Arg Ala Ser Lys Gly Ser Glu
190 195 200 205

Thr Cys Gly Phe Val Asp Glu Arg Gly Leu Tyr Lys Ser Leu Lys Gly
210 215 220

Ala Cys Lys Leu Lys Leu Cys Gly Val Leu Gly Leu Arg Leu Met Asp
225 230 235

Gly Thr Trp Val Ala Met Gln Thr Ser Asn Glu Thr Lys Trp Cys Pro
240 245 250

Pro Asp Gln Leu Val Asn Leu His Asp Phe Arg Ser Asp Glu Ile Glu
255 260 265

His Leu Val Val Glu Glu Leu Val Arg Lys Arg Glu Glu Cys Leu Asp
270 275 280 285

Ala Leu Glu Ser Ile Met Thr Thr Lys Ser Val Ser Phe Arg Arg Leu
290 295 300

Ser His Leu Arg Lys Leu Val Pro Gly Phe Gly Lys Ala Tyr Thr Ile
305 310 315

Phe Asn Lys Thr Leu Met Glu Ala Asp Ala His Tyr Lys Ser Val Arg
320 325 330

Thr Trp Asn Glu Ile Leu Pro Ser Lys Gly Cys Leu Arg Val Gly Gly
335 340 345

Arg Cys His Pro His Val Asn Gly Val Phe Phe Asn Gly Ile Ile Leu
350 355 360 365

Gly Pro Asp Gly Asn Val Leu Ile Pro Glu Met Gln Ser Ser Leu Leu
370 375 380

Gln Gln His Met Glu Leu Leu Glu Ser Ser Val Ile Pro Leu Val His
385 390 395

Pro Leu Ala Asp Pro Ser Thr Val Phe Lys Asp Gly Asp Glu Ala Glu
400 405 410

Asp Phe Val Glu Val His Leu Pro Asp Val His Asn Gln Val Ser Gly
415 420 425

Val Asp Leu Gly Leu Pro Asn Trp Gly Lys Tyr Val Leu Leu Ser Ala
430 435 440 445

Gly Ala Leu Thr Ala Leu Met Leu Ile Ile Phe Leu Met Thr Cys Cys
450 455 460

Arg Arg Val Asn Arg Ser Glu Pro Thr Gln His Asn Leu Arg Gly Thr
465 470 475

Gly Arg Glu Val Ser Val Thr Pro Gln Ser Gly Lys Ile Ile Ser Ser
480 485 490

Trp Glu Ser His Lys Ser Gly Gly Glu Thr Arg Leu
495 500 505

(2) INFORMATION ON SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 Base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: cDNA

(ix) FEATURES:
(A) NAME/KEY: 5'UTR
(B) POSITION: 1..24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTCAACAGCG CACCCACTTC AAGC 24

(2) INFORMATION ON SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 Base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: cDNA

(ix) FEATURES:
(A) NAME/KEY: 5'UTR
(B) POSITION: 1..24

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCTTGTTGAG ATGATGCTTT GACA 24

(2) INFORMATION ON SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 Base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: cDNA

(ix) FEATURES:
(A) NAME/KEY: 5'UTR
(B) POSITION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGTCCTGTGT CTGAACCTGA G 21

(2) INFORMATION ON SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 Base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: cDNA

(ix) FEATURES:
(A) NAME/KEY: 5'UTR
(B) POSITION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTATGGCCTG GGGCGTTTAC A 21

(2) INFORMATION ON SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Peptide

(ix) FEATURES:

(A) NAME/KEY: Peptide
(B) POSITION: 1..41

(ix) FEATURES:
(A) NAME/KEY: Modified site
(B) POSITION: 17
(D) OTHER INFORMATION: /note= "Xaa is Nle"

(ix) FEATURES:
(A) NAME/KEY: Modified site
(B) POSITION: 25
(D) OTHER INFORMATION: /note= "Xaa is Nle"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp Glu Gly
1               5                  10                  15

Xaa Ile Asp Gly Lys Gly Asp Ile Xaa Gly Glu Trp Gly Asn Glu Ile
            20                  25                  30

Phe Gly Glu Ile Ala Glu Phe Leu Gly
        35                  40
```

Claims

1. Process for preparing cancer vaccines which contain autologous tumour cells, characterised in that tumour cells or fibroblasts are cultivated and the cultivated cells are transfected *ex vivo* with a composition which contains the following components:

ai) a DNA molecule which contains one or more sequences expressable in the cells, which code for one or more identical or different immunostimulant polypeptides, or several DNA molecules, containing sequences coding for different immunostimulant polypeptides;

aii) optionally another DNA molecule which is free from sequences coding for a polypeptide which is functionally active in the cell to be transfected;

b) a conjugate between a DNA-binding molecule and an endosomolytically acting agent, selected from the group comprising

i) adenovirus which has a mutation at least in the E4-region,

ii) adenovirus which has one or more other genetic defects in addition to an effect in the E1A-region, or

iii) endosomolytically active peptide;

optionally

c) a DNA-binding molecule, preferably conjugated with an internalising factor which binds to a surface molecule of the cells to be transfected and is internalised into them;

components b) and c) forming a substantially

electroneutral complex with the DNA defined in a),

in that the transfected cells are inactivated so that whilst retaining their ability to express the DNA defined in ai) they lose their ability to divide, whilst in the case of the transfection of fibroblasts the latter are mixed with un-transfected and inactivated tumour cells,

and the cell population is optionally mixed with pharmaceutically acceptable excipients and carriers.

2. Process according to claim 1, characterised in that the DNA defined in ai) is an expression plasmid which contains one or more sequences coding for a cytokine.

3. Process according to claim 2, characterised in that the DNA defined in ai) contains the sequence coding for human interleukin 2.

4. Process according to claim 2, characterised in that the DNA defined in ai) contains the sequence coding for human IFN-γ.

5. Process according to claim 2, characterised in that two plasmids are used as the DNA defined in ai), one of which contains the sequence coding for human interleukin 2 and one of which contains the sequence coding for IFN-γ.

6. Process according to claim 2, characterised in that the DNA defined in ai) contains the sequence coding for human granulocyte-macrophage-colony-stimulating factor (GM-CSF).

7. Process according to claim 1, characterised in that the DNA defined in ai) is an expression plasmid which contains one or more sequences coding for a co-stimulant molecule.

8. Process according to claim 7, characterised in that the DNA contains a sequence coding for the heat-stable antigen.

9. Process according to claim 1, characterised in that the DNA defined in ai) is an expression plasmid which contains one or more sequences coding for a neoantigen.

10. Process according to claim 9, characterised in that the neoantigen is a virus protein or a fragment thereof.

11. Process according to claim 10, characterised in that the virus protein is the rabies glycoprotein.

12. Process according to one of claims 2 to 11, characterised in that, additionally, a plasmid is used as the DNA molecule defined in aii) which is free from sequences, coding for a polypeptide which is functionally active in the cell to be transfected.

13. Process according to claim 1, characterised in that a polylysine, preferably with a chain length of about 200 to 300 lysine groups, is used as the DNA-binding molecule b) and optionally c).

14. Process according to claim 13, characterised in that the polylysine in c) is conjugated with human transferrin as the internalising factor.

15. Process according to claim 13, characterised in that the polylysine in c) is conjugated with human EGF

as internalising factor.

16. Process according to claim 1, characterised in that a conjugate is used as component b) which contains an adenovirus having a defect at least in the E4-region.

17. Process according to claim 16, characterised in that the adenovirus designated dl1014 is used as the E4-mutant.

18. Process according to claim 1, characterised in that, as component b), a conjugate is used which contains an adenovirus with a defect in the E1a-region and which has additionally been inactivated by means of psoralen/UV.

19. Process according to claim 1, characterised in that a conjugate is used as component b), which contains an adenovirus which has been inactivated by means of β-propiolactone.

20. Process according to claim 1, characterised in that a conjugate is used as component b) in which the peptide of sequence SEQ ID NO:7 is ionically bound to polylysine.

21. Process according to claim 1, characterised in that the transfected cells are inactivated with X-rays or gamma-rays.

22. Process according to claim 1, characterised in that the tumour cells are melanoma cells.

23. Process according to claim 1, characterised in that the tumour cells are colon carcinoma cells.

24. Process according to claim 1, characterised in that fibroblasts are transfected and inactivated.

25. Process according to claim 24, characterised in that the fibroblasts are autologous fibroblasts.

26. Process according to claim 24, characterised in that the fibroblasts are cells of a fibroblast cell line.

27. Process according to one of claims 24 to 26, characterised in that the transfected and inactivated fibroblasts are mixed with autologous inactivated tumour cells.

28. Process according to claim 27, characterised in that the tumour cells are melanoma cells.

29. Process according to claim 27, characterised in that the tumour cells are colon carcinoma cells.

30. Cancer vaccines, obtainable by a process according to one of claims 1 to 29.

31. Transfection complex for preparing cancer vaccines, characterised in that it contains the following components:

ai) a DNA molecule which contains one or more sequences expressable in the cells, which code for one or more, identical or different, immunostimulant polypeptides, or a number of DNA molecules containing sequences coding for different immunostimulant polypeptides;

aii) optionally another DNA molecule which is free

from sequences coding for a polypeptide which is functionally active in the cell to be transfected;

b) a conjugate between a DNA-binding molecule and an endosomolytically acting agent, selected from the group comprising

i) adenovirus which has a mutation at least in the E4-region,
ii) adenovirus which has one or more additional genetic defects as well as an effect in the E1A-region, or
iii) endosomolytically active peptide;

optionally
c) a DNA-binding molecule, preferably conjugated with an internalising factor which binds to a surface molecule of the cells to be transfected and is internalised therein,

components b) and c) forming a substantially electroneutral complex with the DNA defined in a).

32. Complex according to claim 31, characterised in that the DNA defined in ai) is an expression plasmid which contains one or more sequences coding for a cytokine.

33. Complex according to claim 29, characterised in that the DNA defined in ai) contains the sequence coding for human interleukin 2.

34. Complex according to claim 32, characterised in that the DNA defined in ai) contains the sequence coding for human IFN-γ.

35. Complex according to claim 32, characterised in that the DNA defined in ai) contains the sequence coding for human GM-CSF.

36. Complex according to claim 32, characterised in that the DNA defined in ai) consists of two plasmids, one of which contains the sequence coding for human interleukin 2 whilst one contains the sequence coding for IFN-γ.

37. Complex according to claim 31, characterised in that the DNA defined in ai) is an expression plasmid which contains one or more sequences coding for a co-stimulant molecule.

38. Complex according to claim 37, characterised in that the DNA contains a sequence coding for the heat-stable antigen.

39. Complex according to claim 31, characterised in that the DNA defined in ai) is an expression plasmid which contains one or more sequences coding for a neoantigen.

40. Complex according to claim 39, characterised in that the neoantigen is a virus protein or a fragment thereof.

41. Complex according to claim 40, characterised in that the virus protein is the rabies glycoprotein.

42. Complex according to one of claims 31 to 41, characterised in that it additionally contains, as the DNA molecule defined in aii), a plasmid which is free from sequences coding for a polypeptide which is functionally active in the cell to be transfected.

43. Complex according to one of claims 31 to 42, characterised in that the DNA contained therein is substantially free from lipopolysaccharides.

44. Complex according to claim 31, characterised in that it contains, as the DNA-binding molecule b) and optionally c), a polylysine, preferably with a chain length of about 200 to 300 lysines.

45. Complex according to claim 44, characterised in that the polylysine in c) is conjugated with human transferrin as internalising factor.

46. Complex according to claim 44, characterised in that the polylysine is conjugated with human EGF as internalising factor.

47. Complex according to claim 31, characterised in that it contains as component b) a conjugate which has an adenovirus with a defect at least in the E4-region.

48. Complex according to claim 47, characterised in that it contains as the E4-mutant the adenovirus designated dl1014.

49. Complex according to claim 31, characterised in that it contains as component b) a conjugate which contains an adenovirus which is inactivated by β-propiolactone.

50. Complex according to claim 31, characterised in that it contains as component b) a conjugate which contains an adenovirus having a defect in the E1a-region, which has additionally been inactivated using psoralen/UV.

51. Complex according to claim 31, characterised in that it contains as component b) a conjugate in which the peptide of sequence SEQ ID NO:7 is ionically bound to polylysine.

52. Human tumour cells transformed with one of the complexes defined in one of claims 31 to 51.

53. Human fibroblasts, transformed with one of the complexes defined in one of claims 31 to 51.

54. Fibroblasts according to claim 53 in admixture with human tumour cells.

55. Pharmaceutical preparation containing cells according to claim 52 or 54 and pharmaceutically acceptable nutrients and excipients.

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Fig. 1

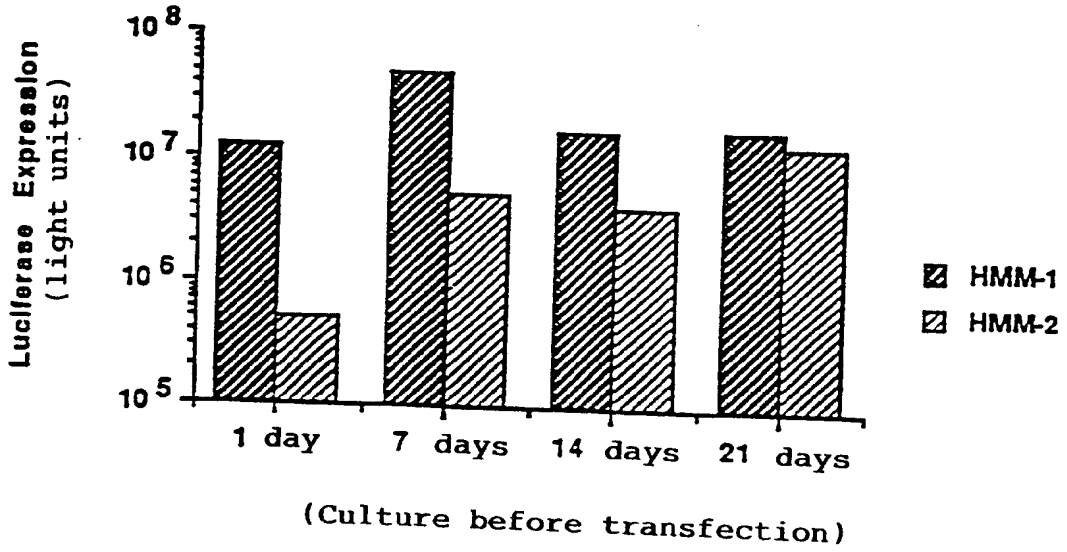

Fig. 2

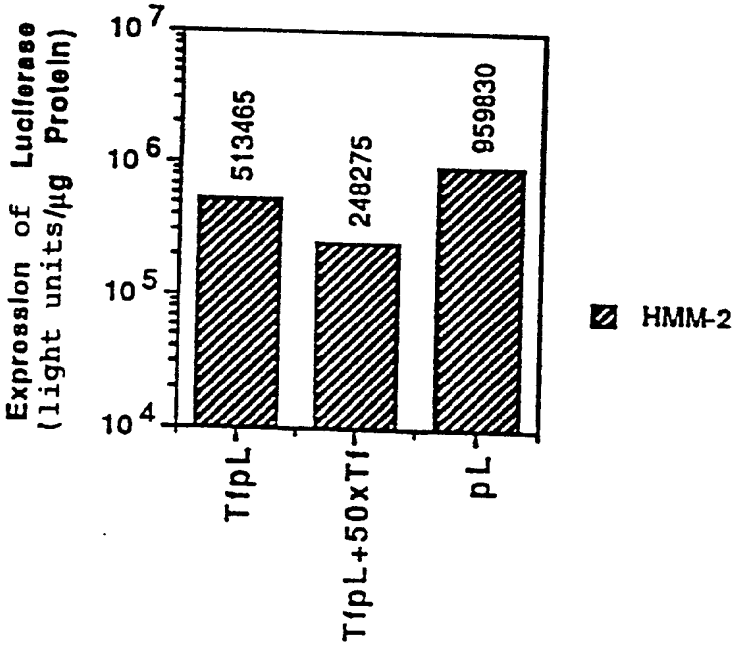
Expression of Luciferase (light units/µg Protein)
10^7
10^6
10^5
10^4
513465
248275
959830
TfpL-
TfpL+50xTf-
pL-
HMM-2

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Fig. 3

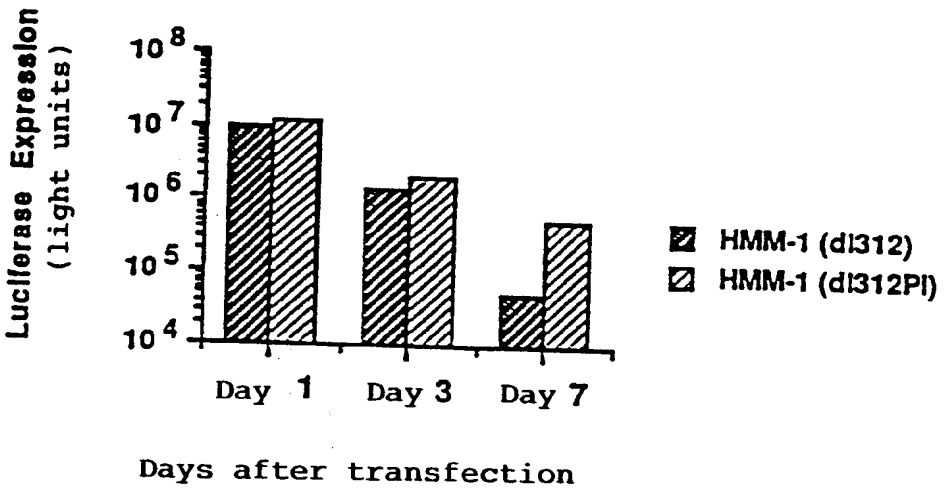

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Fig. 4

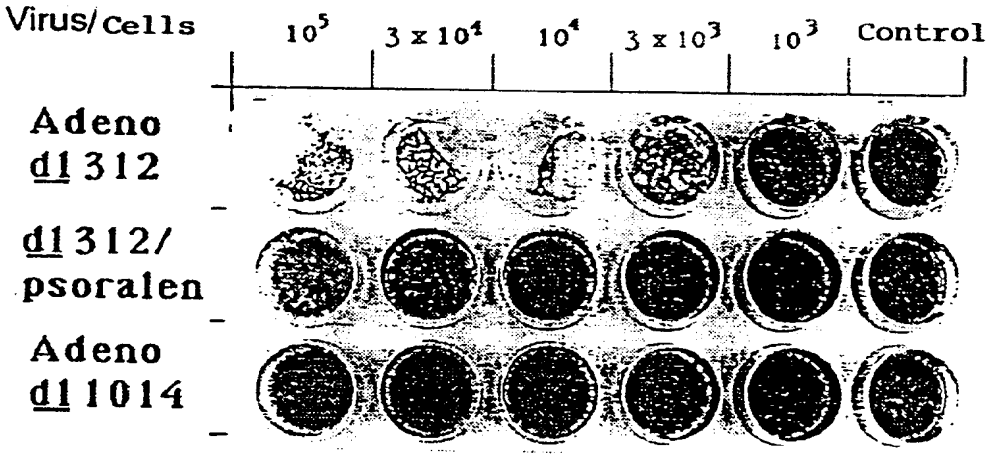

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Fig. 5

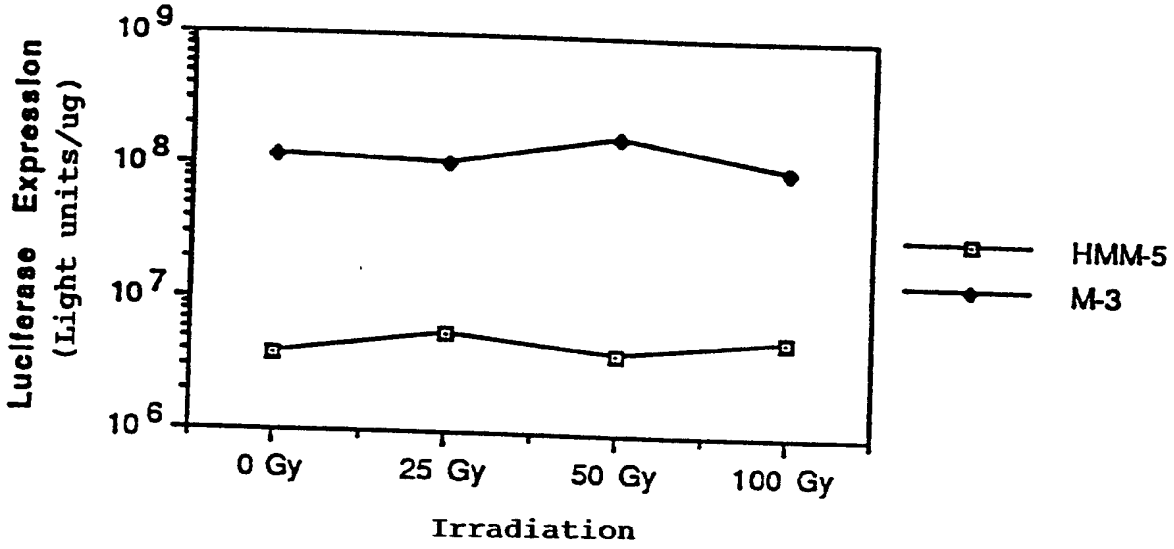

Fig. 6

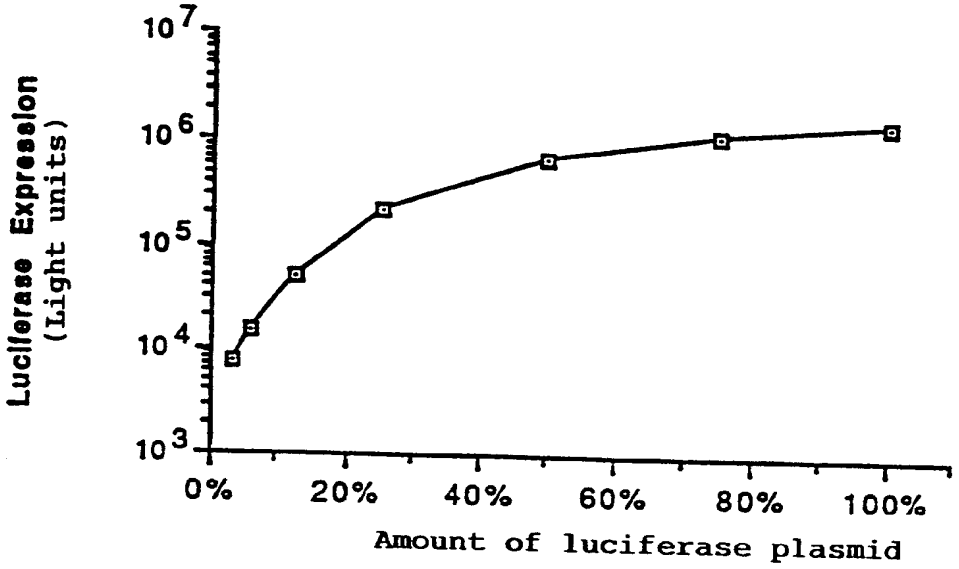

Fig. 7

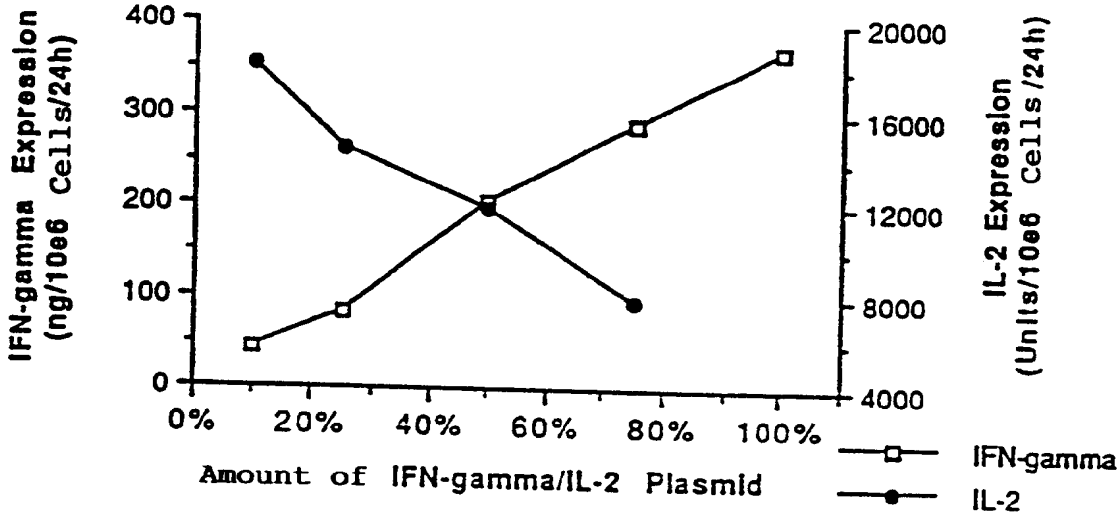

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Fig. 8

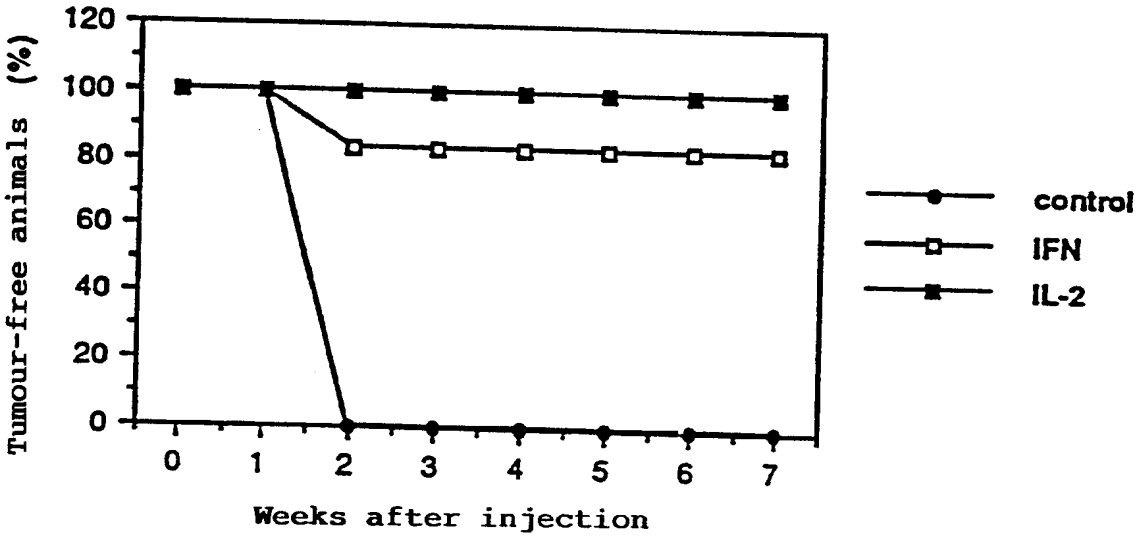

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Fig. 9

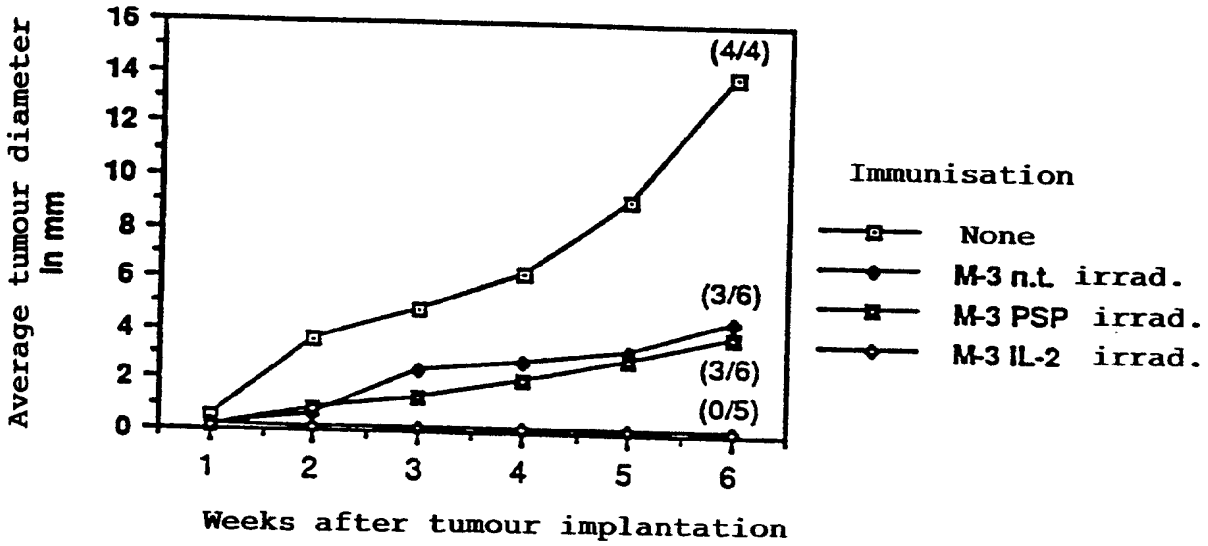

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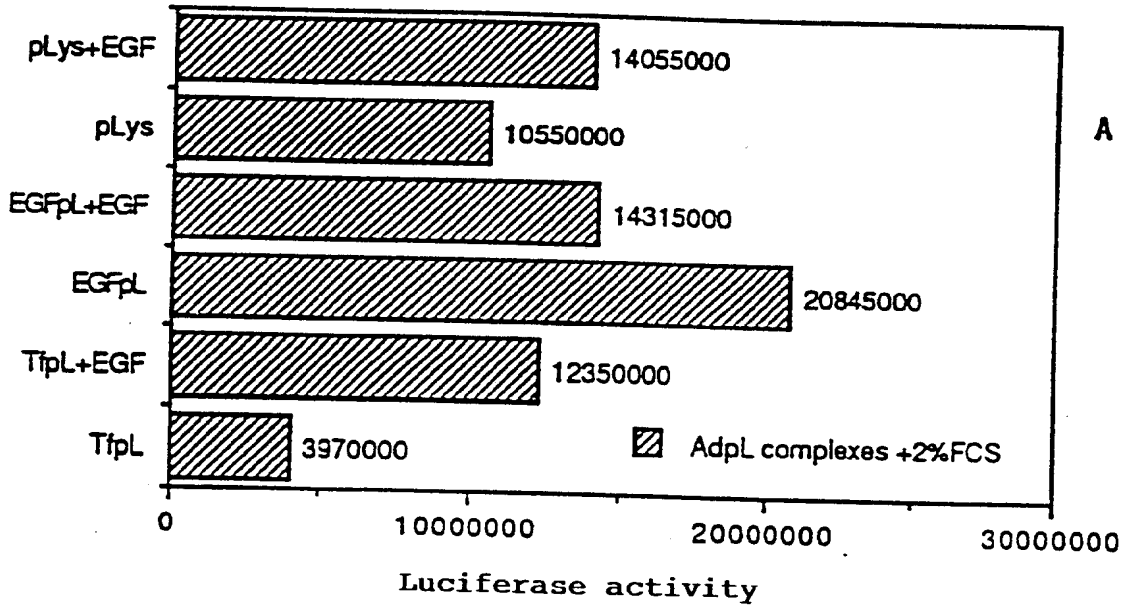
Fig. 10



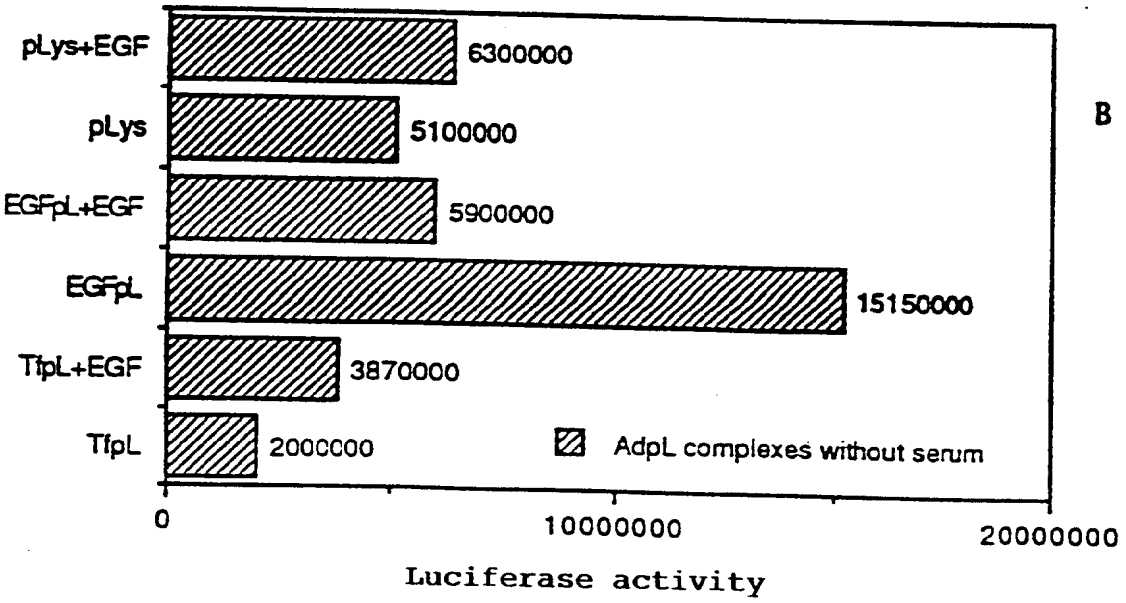


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Fig. 11

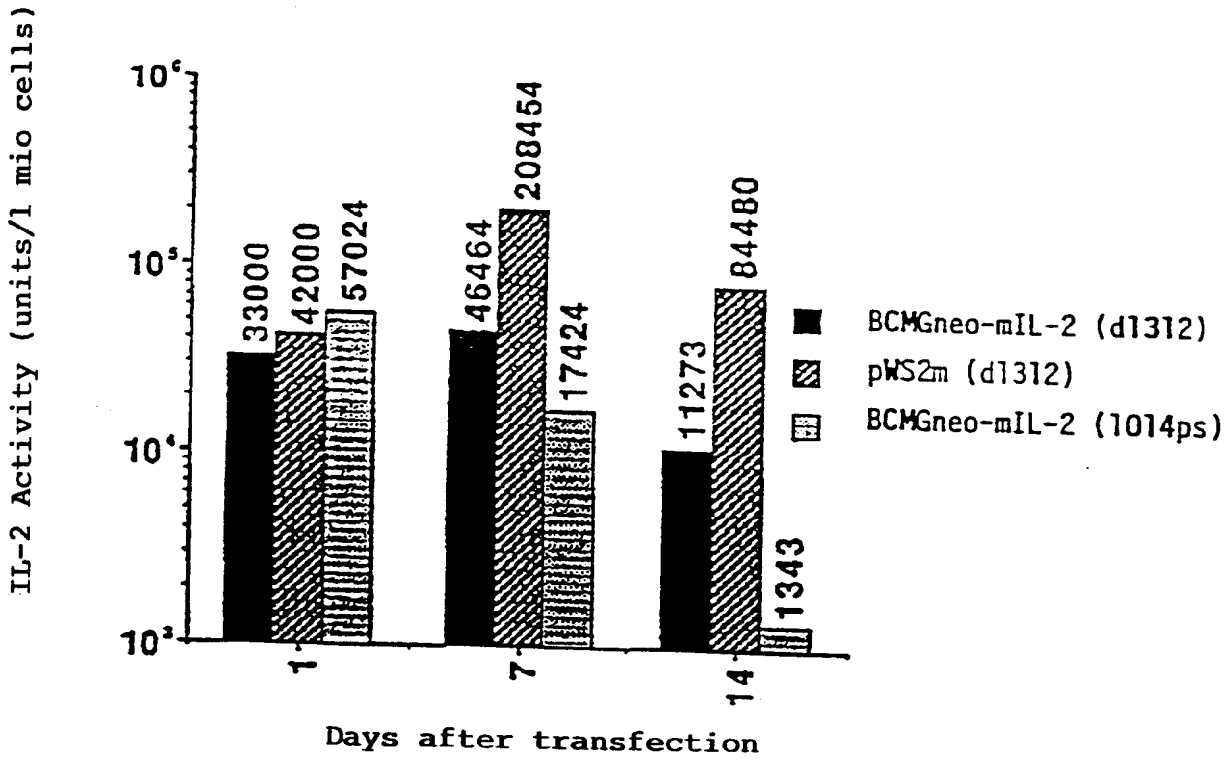

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Fig. 12

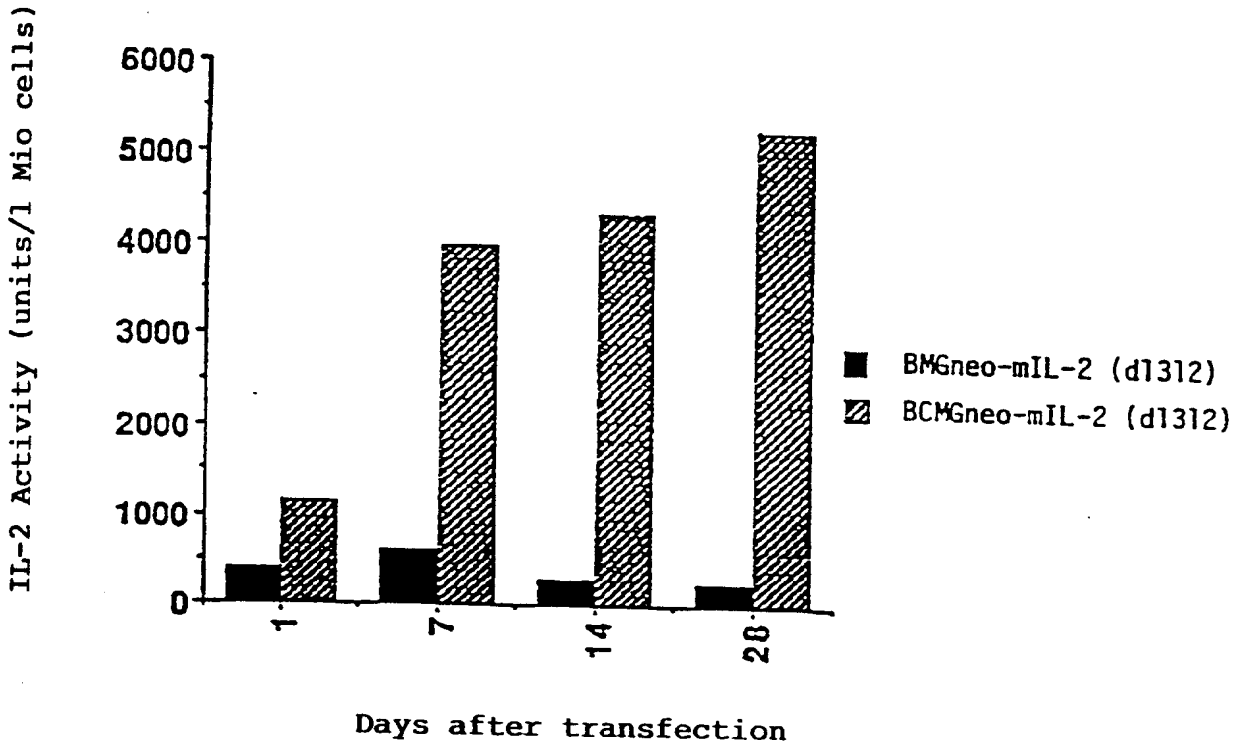

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Fig. 13

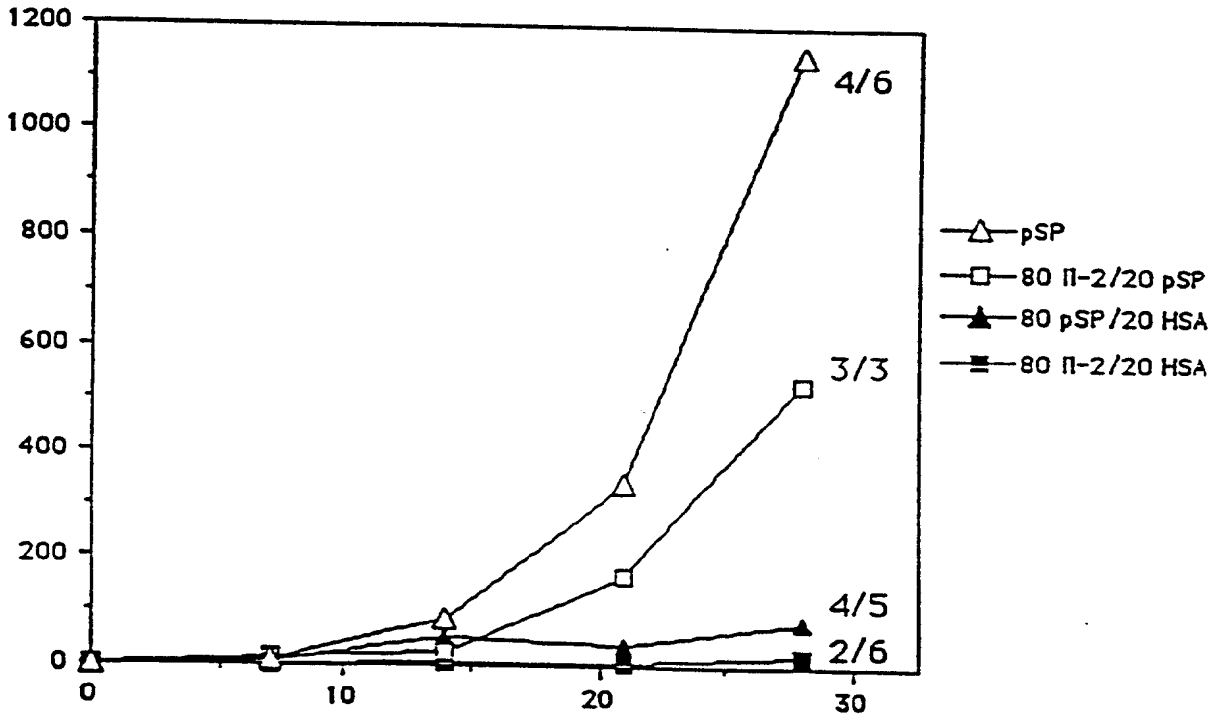

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Fig. 14

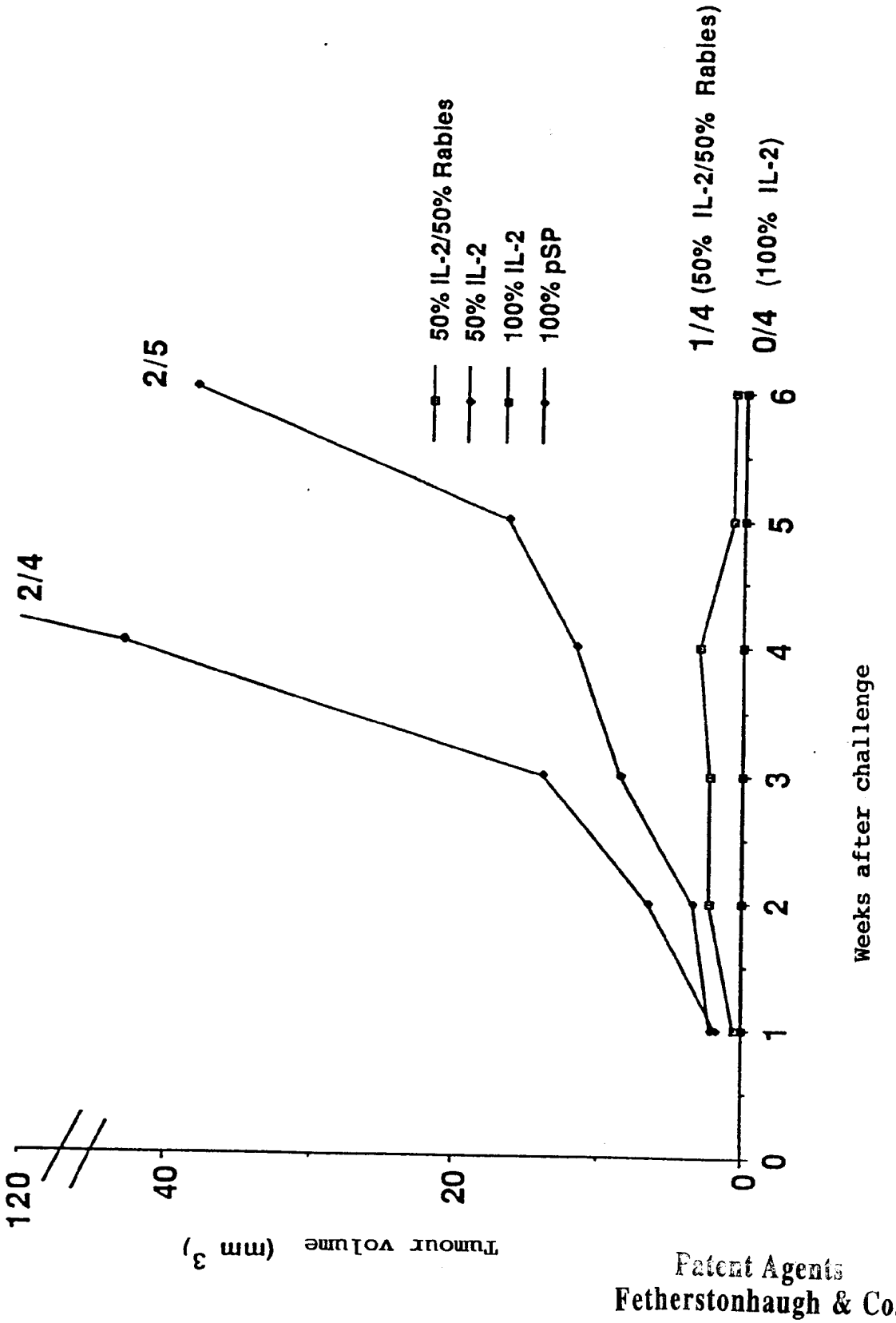

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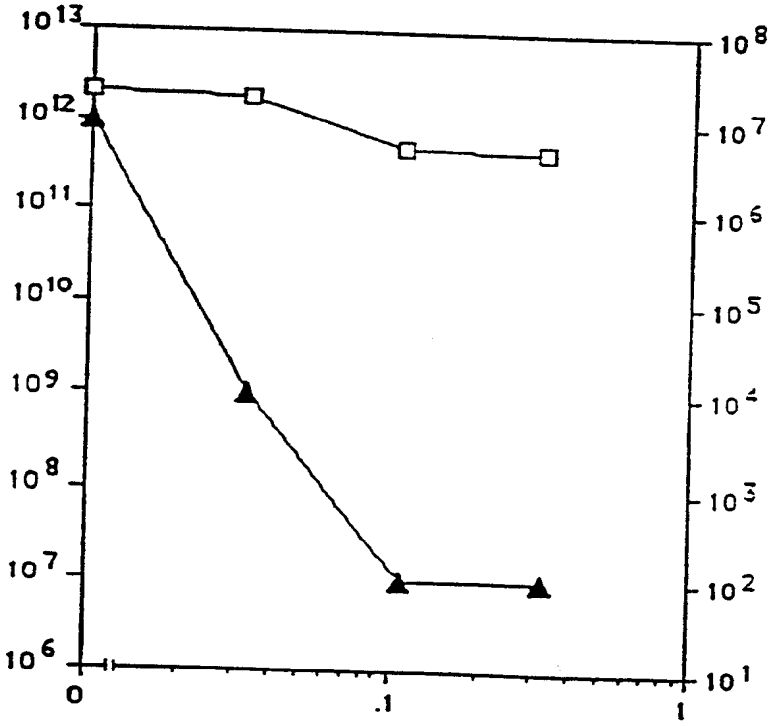
Fig. 15



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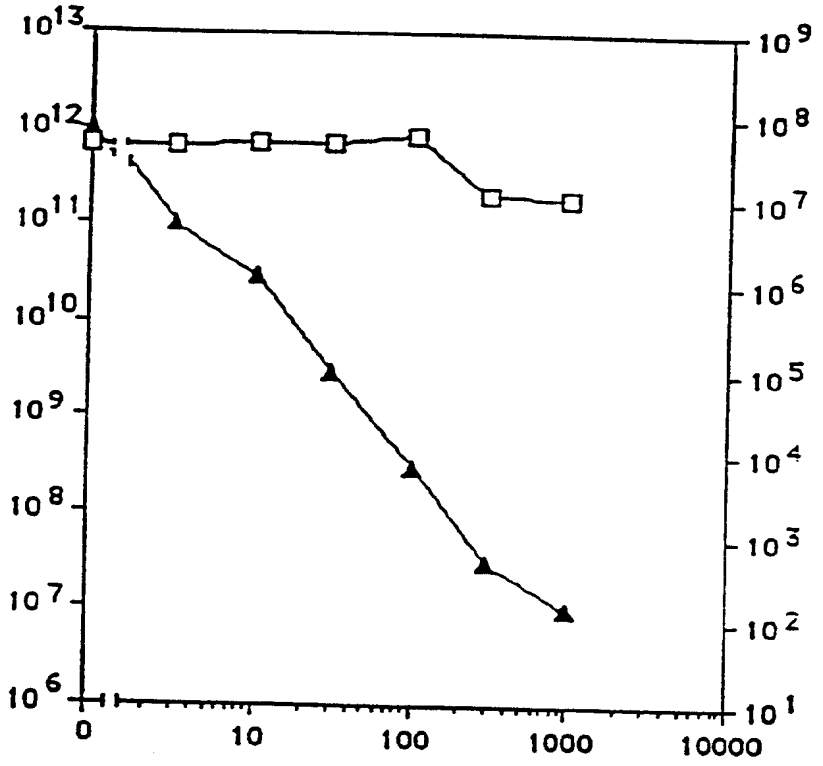

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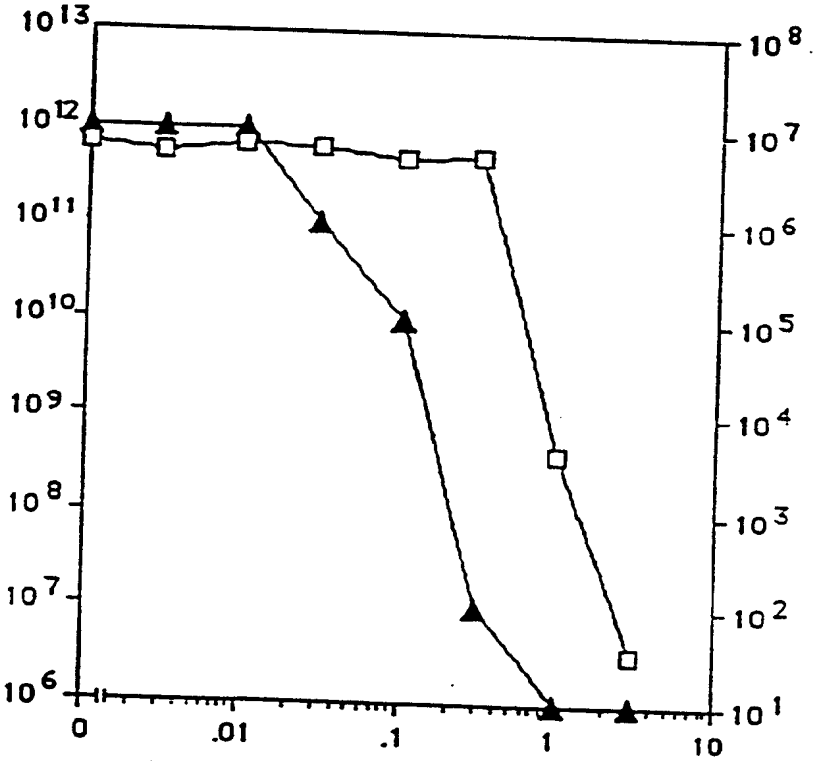

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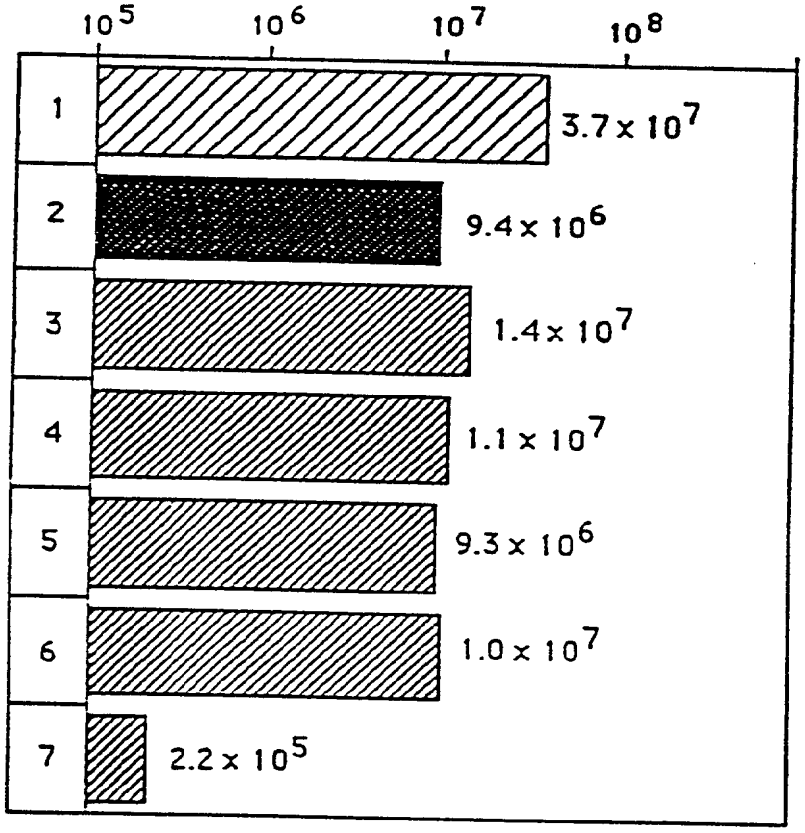

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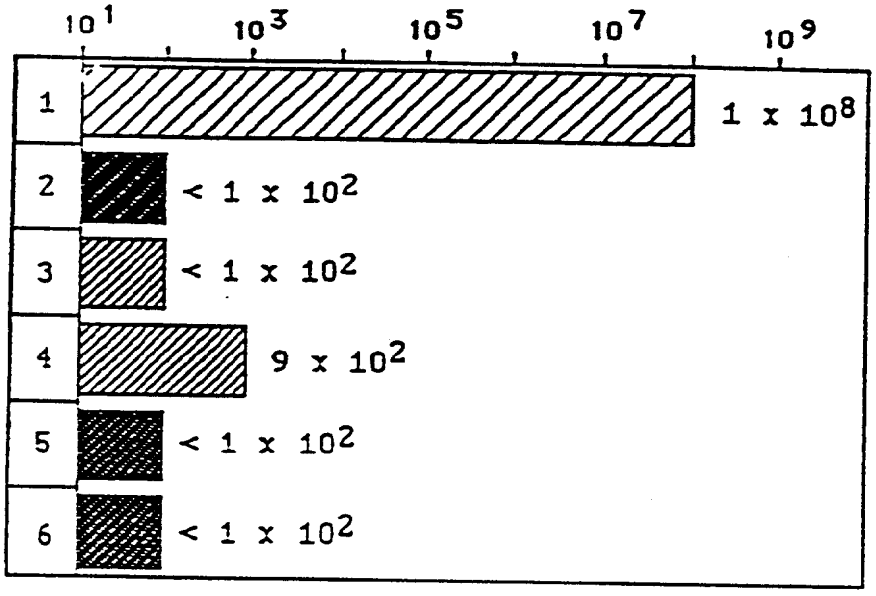

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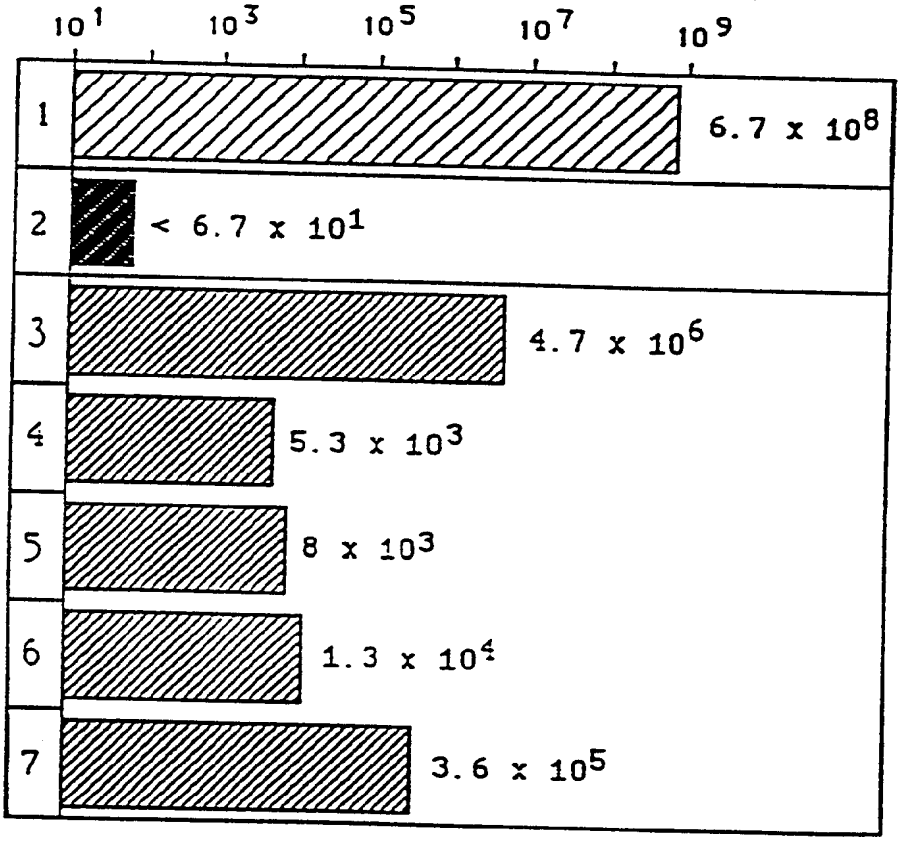

Fig. 21

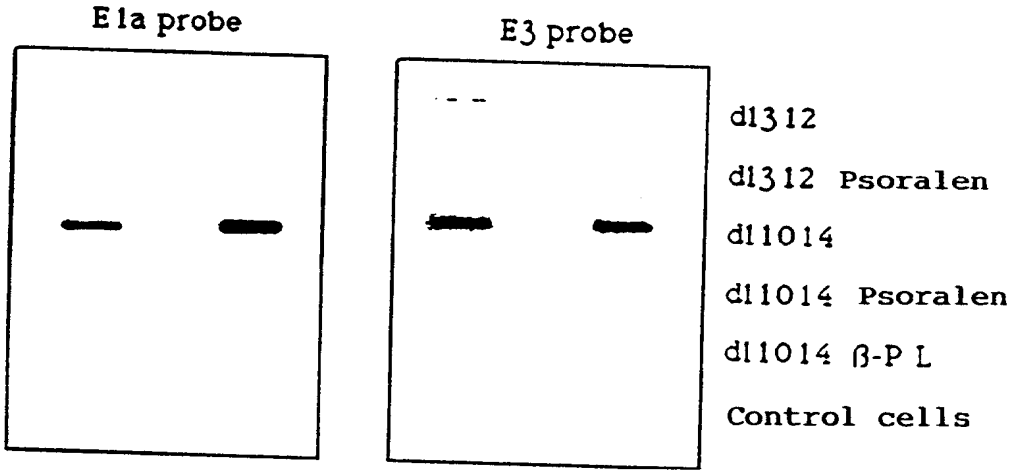

Fig. 22

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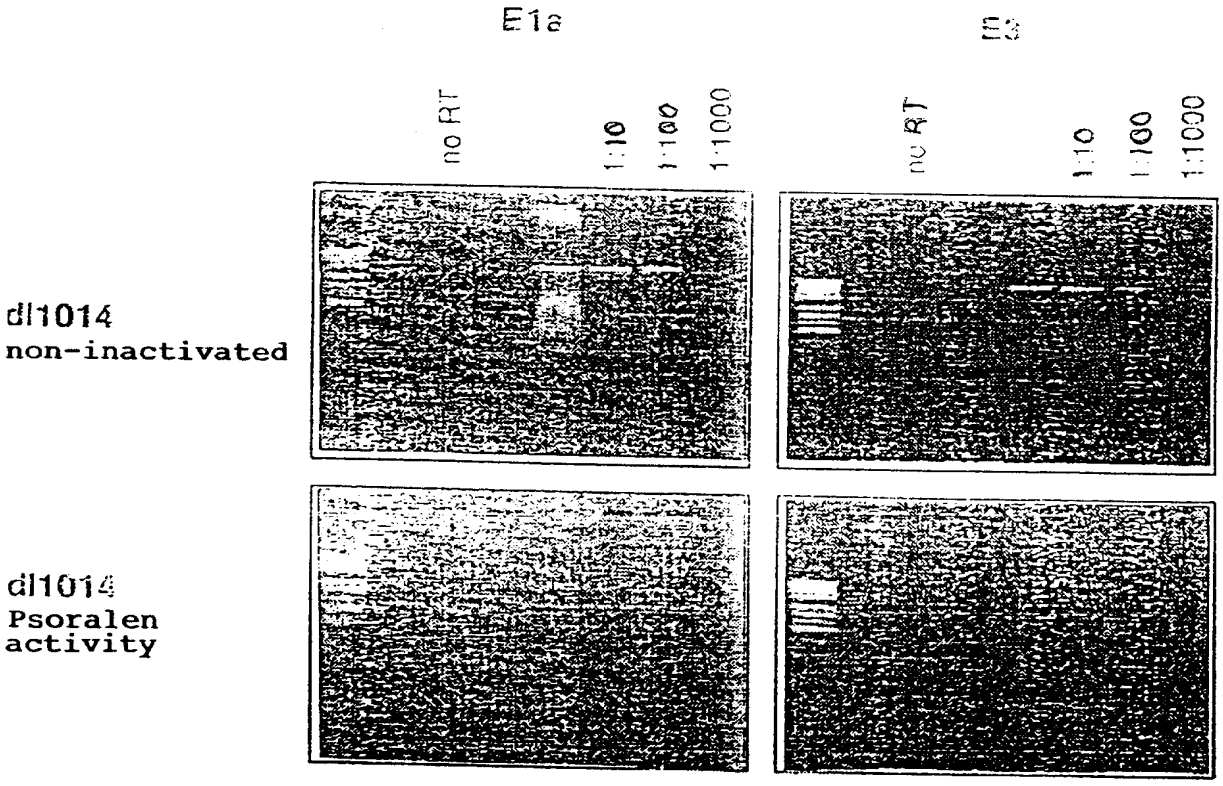

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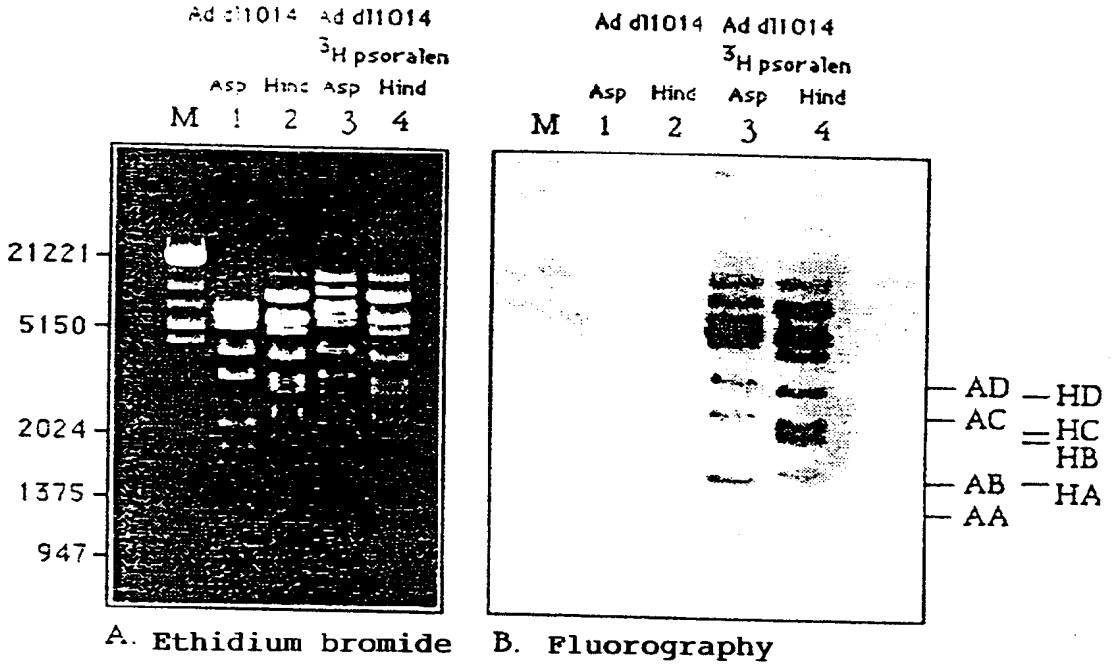
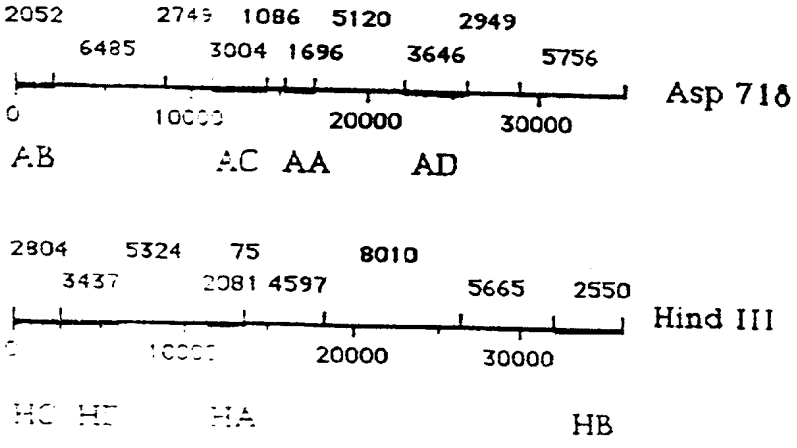
Fig. 23





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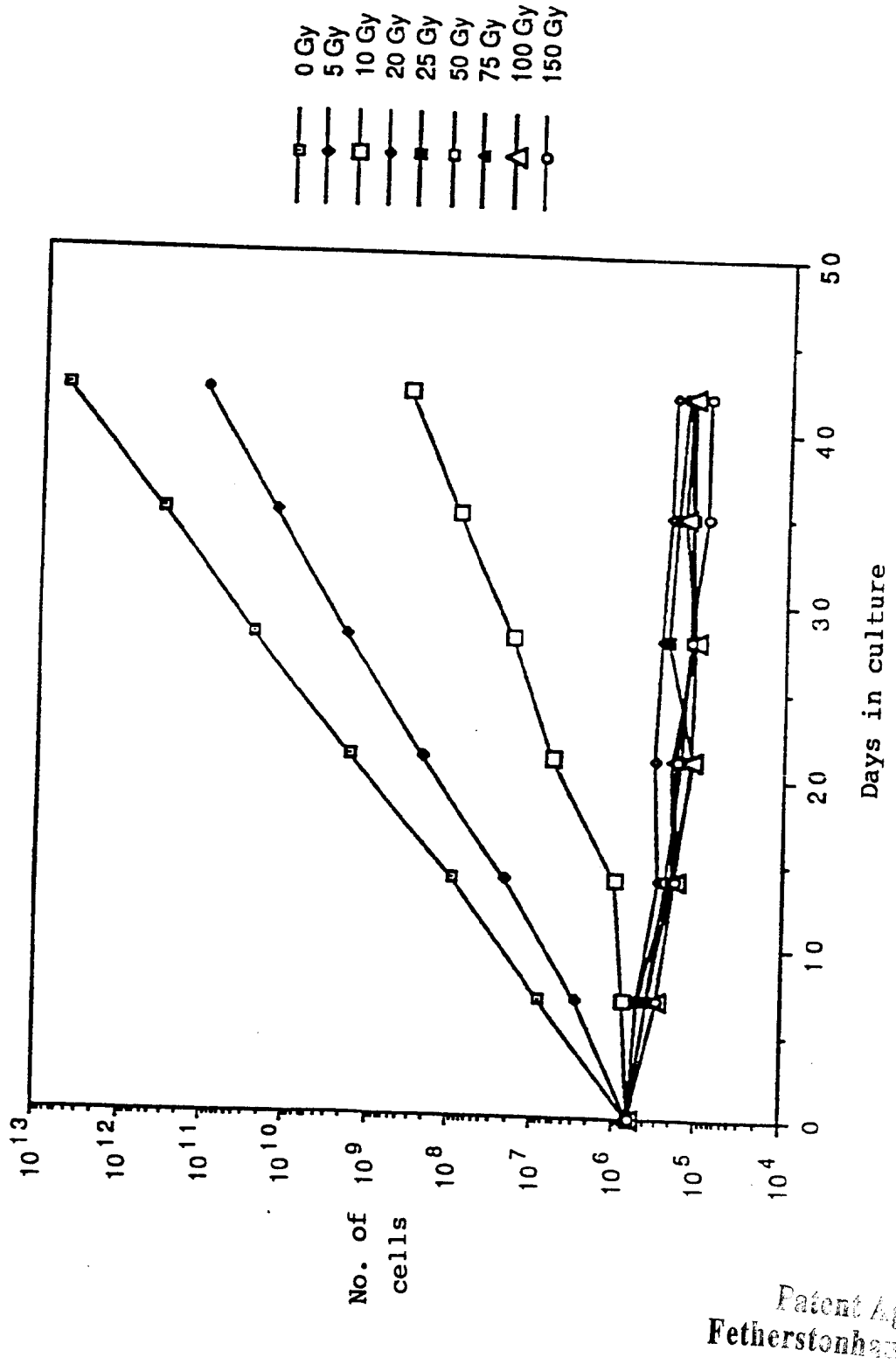

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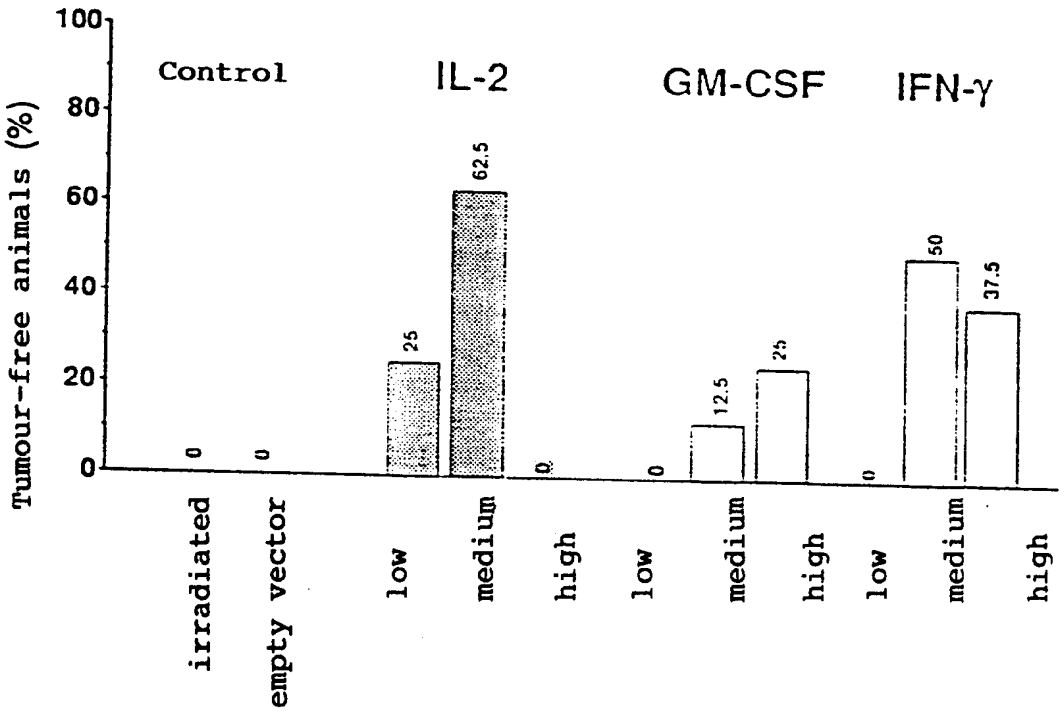

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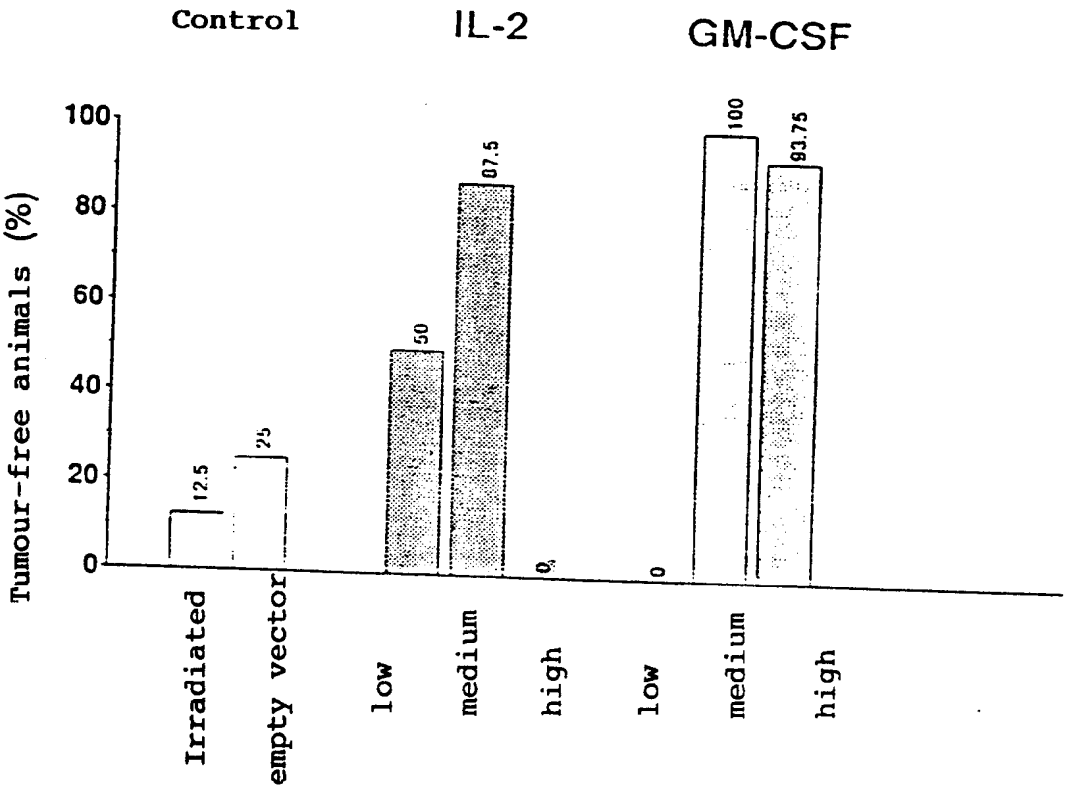

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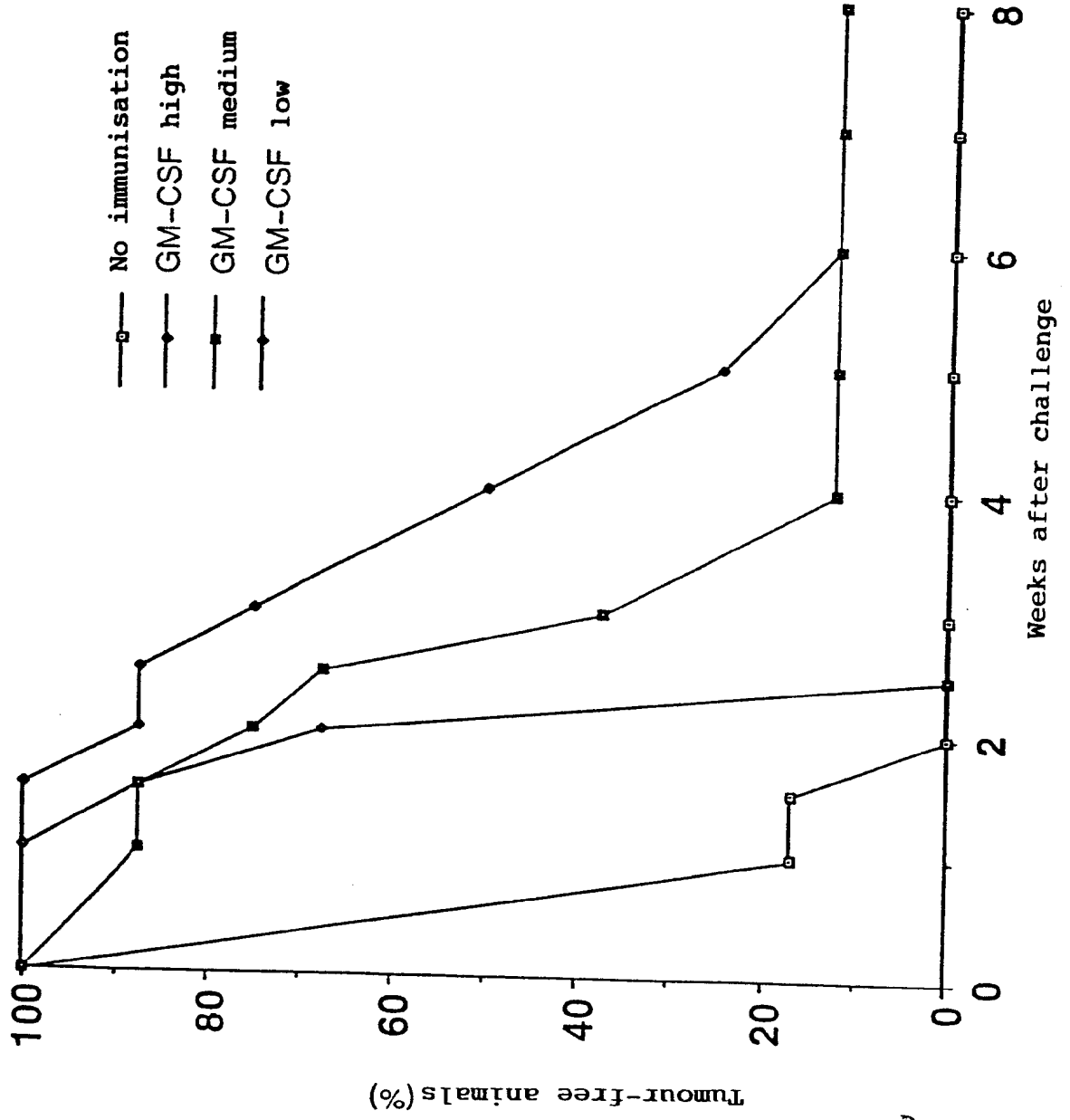

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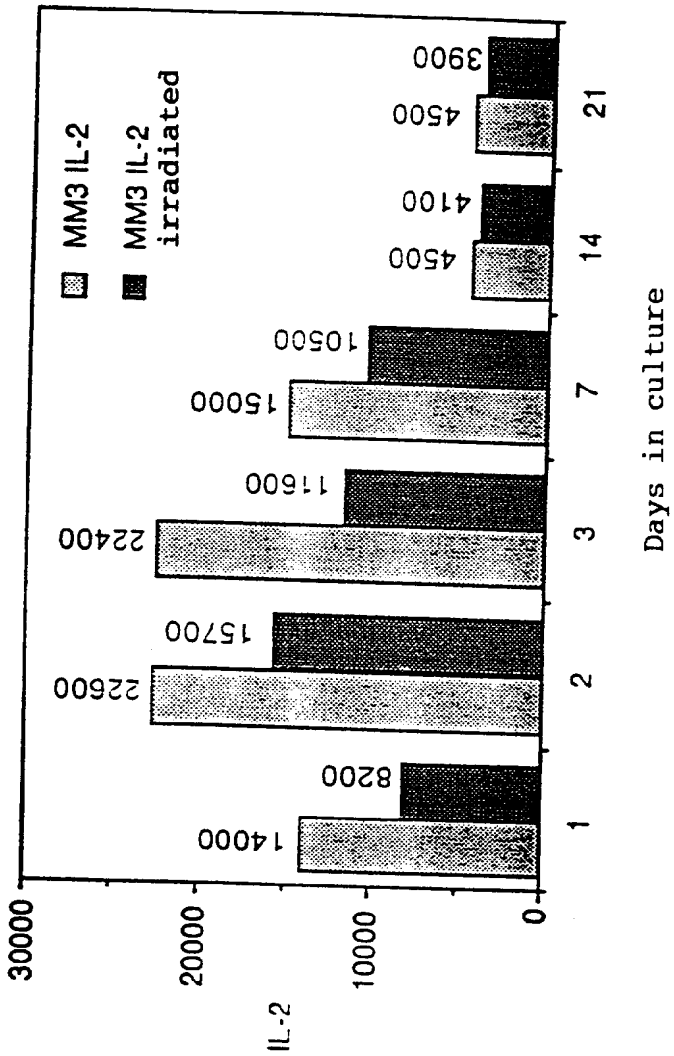

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Fig. 29

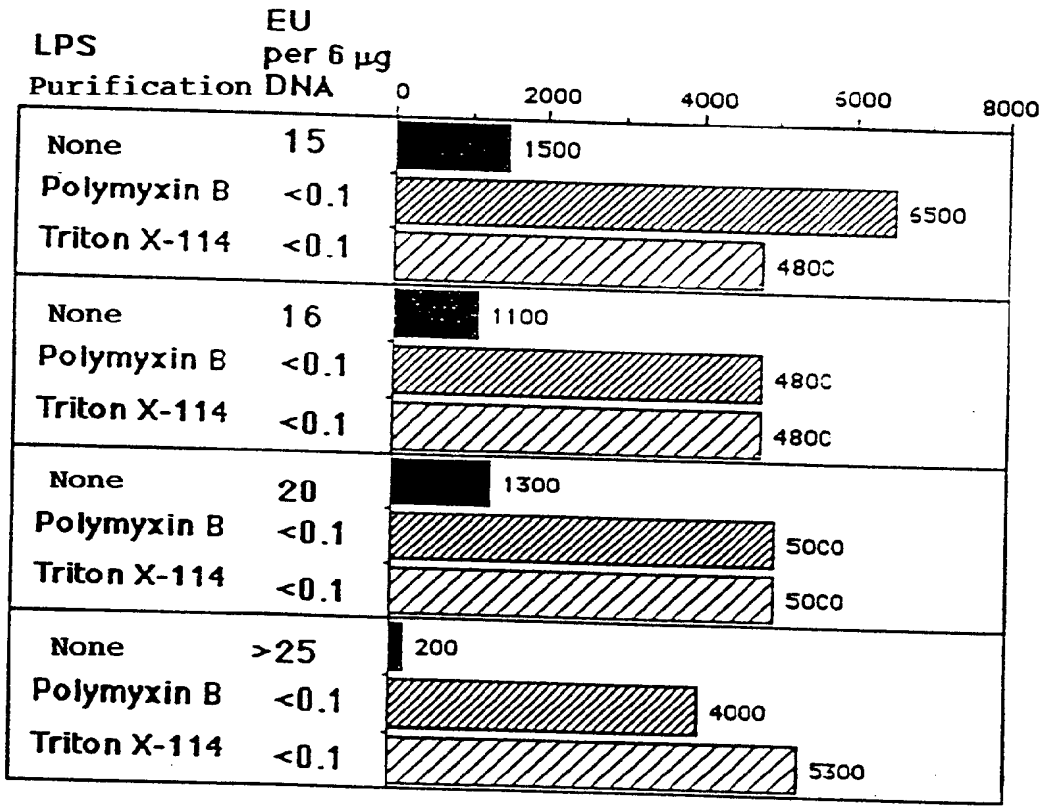

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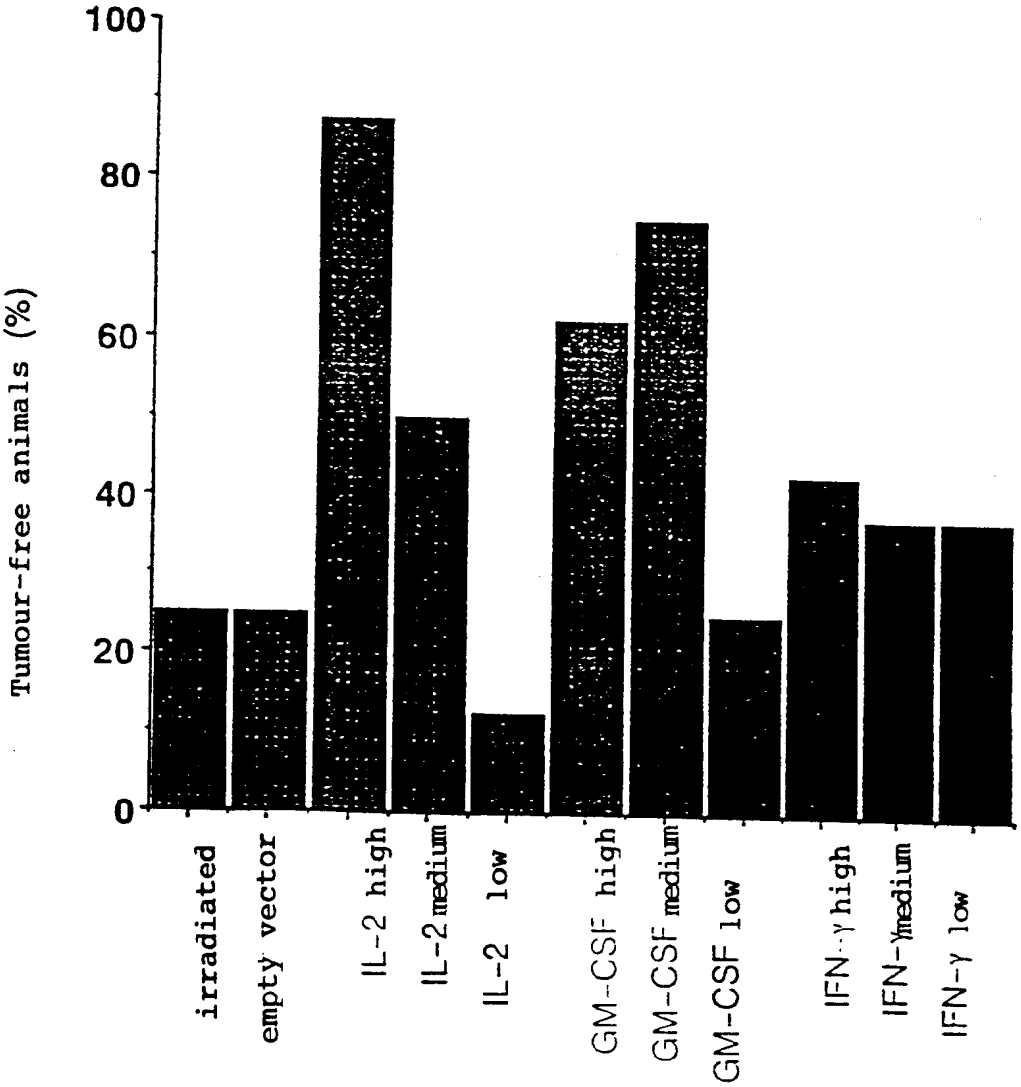
Fig. 30



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